COMPOSITIONS AND METHODS FOR GENE EXPRESSION

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FIELD OF THE INVENTION

The invention relates to the field of nucleotide sequences that mediate one or more functions of IKKα.

BACKGROUND

Proinflammatory cytokines and pathogen associated molecular patterns (PAMPs) activate a classical NF-κB signaling pathway. Upon activation there is inducible degradation of specific inhibitors, IκBs, that retain various NF-κB dimers in the cytoplasm (Ghosh *et al.* (2002) Cell, 109, S81-96). Alternatively, there is an NF-κB signaling pathway induced by IκB Kinase (IKK)α activation of RelB:p52 dimers that induce several chemokine genes (for example lymphotoxin β) needed for organization of secondary lymphoid organs. While the mechanisms of NF-κB activation are well understood (Ghosh *et al.* (2002) Cell, 109, S81-96), the generation of biological specificity by this complex system is more enigmatic (Pomerantz *et al.* (2002)Mol Cell, 10, 693-695).

In vivo analysis revealed that IKKα activates an alternative NF-κB pathway based on processing of NF-κB2/p100 and release of RelB:p52 dimers in response to LTα/β trimers (Dejardin *et al.* (2002) Immunity, 17, 525-535) and other TNF family members (Claudio *et al.* (2002) Nat Immunol, 3, 958-965; Kayagaki *et al.* (2002) Immunity, 17, 515-524). This pathway is required for secondary lymphoid organogenesis and induction of genes involved in this process, but has no apparent role in TNFα-induced functions (Dejardin *et al.* (2002) Immunity, 17, 525-535; Senftleben *et al.* (2001) Science, 293, 1495-1499). However, the mechanisms by which IKKα regulates cytokine-induced gene expression are obscure and controversial (Israel *et al.* (2003) Nature, 423, 596-597).

Therefore, it is important to know more about how RelB:p52 dimers are involved in induction of organogenic chemokines and other important regulatory molecules to

activation of the alternative pathway for diseases associated with alterations in IKKα activity.

SUMMARY OF THE INVENTION

The invention provides nucleotide sequences that mediate one or more functions of IKKα, kits and methods for using these sequences to identify therapeutic compounds that alter IKKα related pathology.

The invention provides an isolated nucleotide sequence comprising 5'-NGGAGANNTG-3' (SEQ ID NO:57); wherein N at position 1 is chosen from G and A, N at position 7 is chosen from T and C, and N at position 8 is chosen from T and C, and wherein the isolated sequence specifically binds with a polypeptide sequence comprising SEQ ID NO:62. In one embodiment, binding with the polypeptide sequence increases transcription of a nucleic acid sequence of interest that is operably linked to 5'-NGGAGANNTG-3' (SEQ ID NO:57); wherein N at position 1 is chosen from G and A, N at position 7 is chosen from T and C, and N at position 8 is chosen from T and C. In another embodiment, the isolated nucleotide sequence does not bind a protein comprising one or more of RelB, RelA, p50, RelB:p50, RelA:p50, and RelA:p52. In another embodiment, the isolated nucleotide sequence does not bind one or more of RelB:Rel, RelA:RelA, p50:p50, RelB:p50, RelA:p50, and RelA:p52 dimers. In a further embodiment, the isolated nucleotide sequence comprises 5'-GGGAGATTTG-3' (SEQ ID NO:59). In yet another embodiment, the isolated nucleotide sequence comprises 5'-GGGAGACCTG-3' (SEQ ID NO:2). In an alternative embodiment, the isolated nucleotide sequence comprises 5'-AGGAGATTTG-3' (SEQ ID NO:60). In yet another embodiment, the isolated nucleotide sequence is a probe, enhancer, and/or promoter. In preferred embodiment, the promoter is chosen from one or more of *Sdf*-1 promoter, *Blc* promoter, *Elc* promoter, and *Slc* promoter. In another embodiment, the isolated nucleotide sequence is chosen from one or more of nonsense sequence, intron, and exon. In yet a further embodiment the polypeptide sequence comprises RelB. Preferably, the polypeptide sequence comprises RelB:p52.

Also provided is a method for identifying one or more test compounds that alters binding of RelB Rel homology domain (RelB RHD) and/or RelB:p52 dimers with a RelBκB sequence, comprising: a) contacting i) the isolated nucleotide sequence of the invention with ii) a polypeptide comprising RelB RHD listed as SEQ ID NO:62 in the

presence and absence of the one or more test compounds; and b) detecting altered specific binding of the nucleotide sequence with SEQ ID NO:62 in the presence of the one or more test compounds compared to in the absence of the one or more test compounds, and c) identifying the one or more test compounds as altering binding of RelB RHD and/or RelB:p52 dimers with a RelBκB sequence. In one embodiment, the method further comprises d) contacting the nucleotide sequence, in the presence of the one or more test compounds, with one or more compositions comprising a polypeptide that comprises RelB RHD listed as SEQ ID NO:62, wherein the composition is chosen from cell extract, cytoplasmic extract, and nuclear extract, wherein the composition is isolated from a mammalian cell comprising IKKα having reduced kinase activity compared to wild type IKKα, and wherein the mammalian cell is treated with one or more IKKα activators chosen from lymphotoxin B (LTβ), B cell activating factor belonging to the TNF family (BAFF), anti-LTβR antibody, and CD40 ligand (CD40L); and e) detecting unaltered specific binding of the isolated nucleotide sequence with the SEQ ID NO:62 in the presence and absence of the one or more test compounds. In one embodiment, the polypeptide comprising RelB RHD is recombinant. In another embodiment, the polypeptide comprising SEQ ID NO:62 is RelB and/or RelB:p52. In a further embodiment, the polypeptide comprising SEQ ID NO:62 is chosen from one or more of RelB and RelB:p52, and is isolated from a mammalian cell treated with one or more IKKα activators chosen from lymphotoxin B (LTβ), B cell activating factor belonging to the TNF family (BAFF), anti-LTβR antibody, and CD40 ligand (CD40L).

While not limiting the invention to any type or source of cell, in one embodiment, the mammalian cell is from a mammal chosen from human, and mouse. In one embodiment, the mammalian cell is *in vivo* and/or *in vitro*. The mammalian cell may be a primary cell and/or from a cell line.

In one embodiment, the method further comprises detecting unaltered binding of the isolated nucleotide sequence to a protein comprising one or more of RelA Rel homology domain (RelA RHD) listed as SEQ ID NO:65, RelA, p50, and RelA:p50, in the presence and absence of the one or more test compounds. Preferably, the polypeptide comprising one or more of RelA Rel homology domain (RelA RHD) comprising SEQ ID NO:65, RelA, p50, and RelA:p50 is recombinant. Alternatively, the polypeptide is chosen from one or more of RelA, p50 and RelA:p50, and is isolated from a cell treated with one or more IKKβ

activators chosen from tumor necrosis factor α (TNFα), interleukin 1β (IL1β), and lipopolysaccharide (LPS).

In one embodiment, the method further comprises detecting unaltered binding of the isolated nucleotide sequence to one or more proteins chosen from a polypeptide comprising one or more of RelA RHD, RelA, p52, and RelA:p52, in the presence and absence of the one or more test compounds. Alternatively, the method further comprises detecting unaltered binding of the isolated nucleotide sequence to one or more proteins chosen from a polypeptide comprising one or more of RelB RHD, RelB, p50, and RelB:p50, in the presence and absence of the one or more test compounds. In yet another alternative, the method further comprises detecting unaltered binding of an isolated nucleotide sequence comprising the consensus-κB sequence 5'-GGGACTTTCC-3' (SEQ ID NO:58) to a polypeptide comprising one or more of RelB RHD listed as SEQ ID NO:62, and RelB in the presence of the one or more test compounds. In preferred embodiment, the method further comprises identifying the one or more test compounds as altering IKKα cellular activity and/or identifying the one or more test compounds as altering symptoms associated with IKKα related pathology.

While not limiting the invention to any method for detection, in one embodiment, the detecting comprises using an array, using electrophoretic mobility shift assay (EMSA), immunoprecipitation, ELISA, footprinting assay, reporter gene assay, and/or optical affinity biosensor system assay and the like. In one embodiment the detecting comprises using a plurality of reaction compartments. Preferably, each of the reaction compartments comprises one test compound. More preferably, the test compound in each of the reaction compartments is different from the test compound in other reaction compartments. In one embodiment, the plurality of reaction compartments comprises a micro-well titre plate. Alternatively, the plurality of reaction compartments comprises at least 48 or at least 96 of the reaction compartments.

Also provided by the invention is a method for identifying one or more test compounds that alters binding of RelB Rel homology domain (RelB RHD) with a RelBκB sequence, comprising: a) contacting i) the isolated nucleotide sequence of the invention, wherein SEQ ID NO:57 is operably linked to a nucleic acid sequence encoding a reporter molecule with ii) a polypeptide comprising RelB Rel homology domain (RelB RHD) listed as SEQ ID NO:62 such that the SEQ ID NO:62 specifically binds with SEQ ID NO:57, wherein the contacting is in the presence and absence of the one or more test compounds; b)

detecting an altered level of expression of the reporter molecule in the presence of the one or more test compounds compared to in the absence of the one or more test compounds, thereby identifying the one or more test compounds as altering binding of RelB Rel homology domain (RelB RHD) with a RelBκB sequence. In one embodiment, the method further comprises: c) contacting the isolated nucleotide sequence, in the presence of the one or more test compounds, with one or more compositions comprising a polypeptide that comprises RelB RHD listed as SEQ ID NO:62, wherein the composition is chosen from cell extract, cytoplasmic extract, and nuclear extract, wherein the composition is isolated from a mammalian cell comprising non-activatable IKKα, and wherein the mammalian cell is treated with one or more IKKα activators chosen from lymphotoxin B (LTβ), B cell activating factor belonging to the TNF family (BAFF), anti-LTβR antibody, and CD40 ligand (CD40L); and d) detecting unaltered binding of the isolated nucleotide sequence with SEQ ID NO:62 in the presence and absence of the one or more test compounds.

In one embodiment, the method further comprises detecting unaltered binding of the isolated nucleotide sequence to one or more proteins chosen from a polypeptide comprising one or more of RelA RHD, RelA, p50, and RelA:p50, in the presence and absence of the one or more test compounds. In another embodiment, the method further comprises detecting unaltered binding of the isolated nucleotide sequence to one or more proteins chosen from a polypeptide comprising one or more of RelA RHD, RelA, p52, and RelA:p52, in the presence and absence of the one or more test compounds. In yet another embodiment, the method further comprises detecting unaltered binding of the isolated nucleotide sequence to one or more proteins chosen from a polypeptide comprising one or more of RelB RHD listed as SEQ ID NO:62, RelB, p50, and RelB:p50, in the presence and absence of the one or more test compounds. In a further comprising detecting unaltered binding of an isolated nucleotide sequence comprising the consensus-κB sequence 5'-GGGACTTTCC-3' (SEQ ID NO:58) to a polypeptide comprising one or more of RelB RHD listed as SEQ ID NO:62, and RelB in the presence of the one or more test compounds. In one embodiment, the method further comprises identifying the one or more test compounds as altering IKKα cellular activity and/or as altering symptoms associated with IKKα related pathology. In a further embodiment, the nucleic acid sequence of interest encodes a reporter molecule. Preferably, the reporter molecule is chosen from one or more of RNA and polypeptide. In one embodiment, the reporter molecule is chosen from one or

more of luciferase, green fluorescent protein, β-galactosidase, human placental alkaline phosphatase, horseradish peroxidase, and chloramphenicol acetyltransferase.

The invention also provides a method for expression of a nucleic acid sequence of interest, comprising: a) providing: i) a cell comprising the isolated nucleotide sequence of the invention, wherein the SEQ ID NO:57 is operably linked to the nucleic acid sequence of interest; and ii) a polypeptide comprising RelB Rel homology domain (RelB RHD) listed as SEQ ID NO:62; b) contacting the cell with the polypeptide such that the SEQ ID NO:62 specifically binds with SEQ ID NO:57, and the nucleic acid sequence of interest is expressed.

Also provided herein is a test compound identified according to any of the invention's methods, such as using an array, using electrophoretic mobility shift assay (EMSA), immunoprecipitation, ELISA, footprinting assay, reporter gene assay, optical affinity biosensor system assay, and/or reporter gene assay and the like.

The invention additionally provides a method for altering symptoms of an IKKα related pathology comprising administering to a mammalian subject one or more compounds that alters binding of RelB Rel homology domain (RelB RHD) with a RelBκB sequence. In one embodiment, the method further comprises observing altered symptoms of the IKKα related pathology. In a preferred embodiment, the symptoms are reduced or increased. The method may comprise observing altered symptoms of the IKKα related pathology.

The invention provides a kit comprising the isolated nucleotide sequences of the invention. In one embodiment, the kit further comprising instructions for binding the isolated nucleotide sequence with a polypeptide comprising RelB RHD listed as SEQ ID NO:62.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows that stromal cell-derived chemokine production requires IKKα. Panel (A) shows Impaired FDC maturation is inherent to the stroma of *Ikkα*$^{AA/AA}$ mice. Top panel: Cryosections of spleen from WT (n = 6) and *Ikkα*$^{AA/AA}$ (n =6) mice, isolated 7 days post-immunization with SRBC, were stained for FDCs (arrows) with FDC-M2 (orange) and anti-B220 (green). Bottom panel: Lethally irradiated WT (n = 6) or *Ikkα*$^{AA/AA}$ (n = 6) mice were reconstituted with *Ikkα*$^{AA/AA}$ or WT bone marrow, respectively. Spleens were isolated and analyzed as above. An FDC network is present in WT mice reconstituted with

Ikkα$^{AA/AA}$ bone marrow, while primarily peri-follicular rings of CD35^{+} immature FDCs are present in *Ikkα*$^{AA/AA}$ reconstituted with *WT* bone marrow. Panel (B) shows impaired B/T cell segregation in *Ikkα*$^{AA/AA}$ spleens. Lethally irradiated WT (n = 3) or *Ikkα*$^{AA/AA}$ (n = 3) mice reconstituted with *Ikkα*$^{AA/AA}$ or WT bone marrow cells were immunized and analyzed as described herein. Impaired B/T cell segregation is intrinsic to the *Ikkα*$^{AA/AA}$ stroma. Panel (C) shows defective chemokine gene expression by *Ikkα*$^{AA/AA}$ spleens. Total splenocytes from naïve and SRBC-immunized (day 2) WT (n = 3) and *Ikkα*$^{AA/AA}$ (n = 3) mice were isolated. RNA was extracted and analyzed by real-time PCR for expression of mRNAs encoding BLC, SLC, ELC and SDF-1 and two of their receptors (CXCR5, CCR7). The results are averages ± SD of three independent experiments normalized to the level of cyclophilin mRNA.

Figure 2 shows IKKα is required for LTβR–induced RelB:p52 nuclear translocation and chemokine expression in splenic stromal cells and myeloid dendritic cells. *Ikkα*$^{AA/AA}$ stromal cells (A) and bone marrow-derived dendritic cells (BMDC) (C) exhibit specific defects in LTβR-induced gene expression. Total RNA was extracted from either WT or *Ikkα*$^{AA/AA}$ stromal cells or BMDC before and after stimulation with 2 μg/ml agonistic anti-LTβR antibody or 20 ng/ml TNFα. Gene expression was analyzed by real-time PCR. Results are averages ± SD of three independent experiments normalized to the level of cyclophilin mRNA. Panels (B, D) show nuclear translocation of NF-κB proteins. Stromal cells (B) and bone marrow-derived dendritic cells (BMDC) (D) were stimulated with either anti-LTβR antibody or TNFα as indicated. At the indicated time points (hrs) after agonist addition, nuclear extracts were prepared and analyzed by immunoblotting for presence of the indicated NF-κB subunits. The levels of histone H2B were examined to control for loading and proper cell fractionation. Contamination with cytoplasmic proteins was monitored by blotting with anti-actin antibody.

Figure 3 shows that IKKα is required for recruitment of RelB to the *Blc*, *Sdf-1*, *Elc* and *Slc* promoters. Primary cultures of stromal cells (A) and bone marrow-derived dendritic cells (BMDC) (B) from WT and *Ikkα*$^{AA/AA}$ mice were left unstimulated or stimulated with TNFα (T) or anti-LTβR (L). At the indicated time points (hrs) the cells were collected and recruitment of RelA, RelB and the large subunit of RNA polymerase (Pol II) to the indicated promoter regions was examined by ChIP experiments.

Figure 4 shows that the *Blc* and *Elc* promoters contain a unique κB site that is selectively recognized by RelB:p52 dimers. Panel (A) shows the sequence of the 700 bp region covering the proximal *Blc* promoter, contained within the ChIP primer set. The RelB:p52-selective κB site and the TATA box are highlighted. The sequence contained within Probe 1 is indicated by the brackets. Panel (B) shows DNA binding analysis. The different probes were incubated with two different amounts (250 and 500 ng) of the indicated NF-κB dimers and DNA binding was analyzed by EMSA. Panel (C) shows the sequences of the different κB sites 5'-GGGACTTTCC-3' (SEQ ID NO:58), 5'-GGGAGATTTG-3' (SEQ ID NO:59), AGGAGATTTG-3' (SEQ ID NO:60), and 5'-GGGATTTCCC-3' (SEQ ID NO:61).

Figure 5 shows selective, IKKα-dependent, activation of the *Blc* and *Elc* promoters by LTβR engagement and a model explaining these results. Panels (A-B) show that engagement of LTβR selectively induces *Blc*-κB and *Elc*-κB binding activities. WT and IKKα-defective MEFs (A) and bone marrow-derived dendritic cells (BMDC) (B) were left unstimulated or stimulated with either TNFα or anti-LTβR for the indicated times. Nuclear extracts were prepared and incubated with ^{32}P-labeled probes corresponding to the consensus κB site (NF-κB) or the *Blc*-κB and *Elc*-κB sites. DNA binding activity was analyzed by EMSA. NF-1 DNA binding activity was measured as an internal control. Panel (C) shows functional analysis of the different κB sites in the *Blc* and *Elc* promoters. Triple repeats of the consensus κB (conκB), *Blc*-κB and a mutant *Blc*-κB (m*Blc*-κB) site were cloned upstream to a minimal SV40 promoter (pGL3-Promoter vector, Promega). In addition, the *Blc* (+12 to -688) and *Elc* (+530 to -320) promoter regions were cloned upstream to a luciferase reporter (pGL3-Basic vector, Promega). To determine the importance of the *Blc*-κB site, it was converted by site directed mutagenesis either to an inactive mutant version (mκB) or the consensus κB (conκB) site. The different plasmids were transfected into WT and *Ikkα*$^{-/-}$ MEFs. After 6 hrs with TNFα or anti-LTβR, luciferase activity was determined. The results are averages ± SD of three independent experiments normalized to β-galactosidase activity produced by a cotransfected β-galactosidase expression vector.

Figure 6 shows an exemplary model explaining selective gene activation by the two NF-κB signaling pathways. Engagement of TNF-R1 results in activation of the canonical NF-κB signaling pathway which depends on IKKβ and IKKγ. This pathway leads to

degradation of IκB and presence of RelA:p50 and RelB:p50 dimers in the nucleus. RelA:p50, and to a lesser extent RelB:p50, are recruited and lead to activation of the *Vcam-1*, *Tnfα* and *IκBα* genes. The RelA:p50 dimer is essentially not recruited to the *Sdf-1*, *Blc*, *Slc* and *Elc* promoters. By contrast, engagement of LTβR results in robust activation of the alternative pathway which depends on IKKα. This pathway leads to processing of p100 and the nuclear translocation of RelB:p52 dimers. These dimers are recruited to the *Sdf-1*, *Blc*, *Slc* and *Elc* promoters leading to their activation. LTβR engagement can also lead to modest activation of the canonical pathway with all of its sequelae. RelB:p50 dimers may make a small contribution to activation of both classes of genes.

Figure 7 shows the nucleotide sequence (SEQ ID NO:1) of the *sdf-1* gene promoter. The sequence 5'-GGGAGACCTG-3' (SEQ ID NO:2) which binds to NFκ-B2/p52 is higlighted in bold.

Figure 8 shows an exemplary amino acid sequence (SEQ ID NO:3) of human NFκ-B2 (p49p100) (GenBank accession number NM 002502).

Figure 9 shows an exemplary nucleotide sequence (SEQ ID NO:4) encoding human NFκ-B2 (p49/p100) (GenBank accession number NM 002502).

Figure 10 shows an exemplary amino acid sequence (SEQ ID NO:5) of mouse NFκ-B2 (p49/p100) (GenBank accession number BC027423).

Figure 11 shows an exemplary nucleotide sequence (SEQ ID NO:6) encoding mouse NFκ-B2 (p49/p100) (GenBank accession number BC027423).

Figure 12 shows the 3' untranslated terminal repeat of an exemplary Rhesus monkey NFκ-B2 gene (SEQ ID NO:7) (GenBank accession number AY186590).

Figure 13 shows an exemplary amino acid sequence (SEQ ID NO:8) of mouse RelB (GenBank accession number A42023).

Figure 14 shows an exemplary amino acid sequence (SEQ ID NO:9) of mouse RelB (GenBank accession number M83380).

Figure 15 shows an exemplary nucleotide sequence (SEQ ID NO:10) encoding mouse RelB (GenBank accession number M83380).

Figure 16 shows an exemplary amino acid sequence (SEQ ID NO:11) of *Xenopus Laevis* RelB (GenBank accession number D63332).

Figure 17 shows an exemplary nucleotide sequence (SEQ ID NO:12) encoding Xenopus Laevis RelB (GenBank accession number D63332).

Figure 18 shows an exemplary amino acid sequence (SEQ ID NO:13) of mouse NFκ-B1, also known as p105 (GenBank accession number NM 008689), which is processed to p50 (SEQ ID NO:123) (GenBank accession number NP_032715). Figure 19 shows an exemplary nucleotide sequence (SEQ ID NO:14) encoding mouse NFκ-B1, also known as p105, (GenBank accession number NM 008689), which is processed to p50 (SEQ ID NO:123) (GenBank accession number NP_032715).

Figure 20 shows an exemplary amino acid sequence (SEQ ID NO:15) of mouse RelA (GenBank accession number M61909).

Figure 21 shows an exemplary nucleotide sequence (SEQ ID NO:16) encoding mouse RelA (GenBank accession number M61909).

Figure 22 shows an exemplary amino acid sequence (SEQ ID NO:17) (GenBank # AAC51671 and GenBank # AAC51671) of human IKKα.

Figure 23 shows an exemplary nucleotide sequence (SEQ ID NO:18) (GenBank # AF009225) encoding human IKKα.

Figure 24 shows an exemplary amino acid sequence (SEQ ID NO:19) (NIH # NP-031726) of mouse IKKα.

Figure 25 shows an exemplary nucleotide sequence (SEQ ID NO:20) (NIH # NM-007700) of mouse IKKα.

Figure 26 shows that impaired FDC maturation and chemokine production in Stromal cell-derived requires IKKα̃ .

Panel (A) shows absence of mature FDC network in *Ikkα*$^{AA/AA}$ mice. Cryosections of spleen from WT (n = 6) and *Ikkα*$^{AA/AA}$ (n =6) mice, isolated 7 days post-immunization with SRBC, were stained for FDCs (arrows) with FDC-M2 (orange) and anti-B220 (green).

Panel (B) shows impaired FDC maturation is inherent to the *Ikkα*$^{AA/AA}$ stroma. Lethally irradiated WT (n = 6) or *Ikkα*$^{AA/AA}$ (n = 6) mice were reconstituted with *Ikkα*$^{AA/AA}$ or WT bone marrow, respectively. Spleens were isolated 7 days after immunization with SRBC, cryosectioned and stained with anti-CD35. An FDC network is present in WT mice reconstituted with *Ikkα*$^{AA/AA}$ bone marrow, while primarily peri-follicular rings of CD35^{+} immature FDCs are present in *Ikkα*$^{AA/AA}$ mice reconstituted with WT bone marrow.

Panel (C) shows impaired B/T cell segregation in *Ikkα*$^{AA/AA}$ spleens. Lethally irradiated WT (n = 6) or *Ikkα*$^{AA/AA}$ (n = 6) mice reconstituted with *Ikkα*$^{AA/AA}$ or WT bone marrow cells were immunized and analyzed as above using anti-CD5 (to recognize T cells) and anti-B220 (to

recognize B cells). Impaired B/T cell segregation is intrinsic to the *Ikkα*$^{AA/AA}$ stroma. Panel (D) shows defective chemokine gene expression in *Ikkα*$^{AA/AA}$ spleens. Total splenocytes from naïve and SRBC-immunized (day 2) WT (n = 6) and *Ikkα*$^{AA/AA}$ (n = 6) mice were isolated. RNA was extracted and analyzed by RT PCR for expression of mRNAs encoding BLC, SLC, ELC and SDF-1 and two of their receptors (CXCR5, CCR7). The results are averages ± SD of three independent experiments normalized to the level of cyclophilin mRNA.

Figure 27 shows that IKKα is required for LTβR–induced RelB:p52 nuclear translocation and chemokine expression in splenic stromal cells and myeloid dendritic cells. Panel (A) shows *Ikkα*$^{AA/AA}$ stromal cells and (C) bone marrow-derived dendritic cells (BMDC) exhibit specific defects in LTβR-induced gene expression. Total RNA was extracted from either WT or *Ikkα*$^{AA/AA}$ stromal cells or BMDC before and after stimulation with 2 μg/ml agonistic anti-LTβR antibody or 20 ng/ml TNFα. Gene expression was analyzed by Real Time-PCR. Results are averages ± SD of three independent experiments normalized to the level of cyclophilin mRNA.

Panels (B, D) shows nuclear translocation of NF-κB proteins. Stromal cells as in Panel (B) and BMDC as in Panel (D) were stimulated with either anti-LTβR antibody or TNFα as indicated. At the indicated time points (hrs), nuclear extracts were prepared and analyzed by immunoblotting for presence of the indicated NF-κB subunits. The levels of histone H2B were examined to control for loading and proper cell fractionation. Contamination with cytoplasmic proteins was monitored by blotting with anti-actin antibody (not shown).

Figure 28 shows that IKKα is required for recruitment of RelB to the *Blc*, *Sdf-1*, *Elc* and *Slc* promoters. Primary cultures of stromal cells Panel (A) and bone marrow-derived dendritic cells (BMDC) (B) from WT and *Ikkα*$^{AA/AA}$ mice show that when left unstimulated or stimulated with TNFα (T) or anti-LTβR (L). At the indicated time points (hrs) the cells were collected and recruitment of RelA, RelB and the large subunit of RNA polymerase (Pol II) to the indicated promoter regions were examined by ChIP experiments.

Figure 29 shows that The *Blc* and *Elc* promoters contain a unique κB site that is selectively recognized by RelB:p52 dimers.

Panel (A) shows an exemplary sequence of the 700 bp region covering the proximal *Blc* promoter, contained within the ChIP primer set. A RelB-selective κB site and the TATA box are highlighted. The sequence contained within Probe 1 is indicated by the brackets

and is underlined. Panel (B) shows an exemplary DNA binding analysis. The different probes were incubated with two different amounts (250 and 500 ng) of the indicated NF-κB dimers and DNA binding was analyzed by EMSA. Note that the NF-κB subunits are not the full-length proteins, thus giving rise to complexes with different electrophoretic mobilities. Panel (C) shows exemplary sequences of the different κB sites used in these experiments.

Figure 30 shows that selective, IKKα-dependent, activation of the *Blc* and *Elc* promoters by LTβR engagement and IKKα-dependent induction of *Rxra*, *Irf3* and *Baff* mRNAs. Panels (A-B) shows an exemplary engagement of LTβR selectively induces *Blc*-κB and *Elc*-κB binding activities. WT and IKKα-defective MEFs (A) and bone marrow-derived dendritic cells (BMDC) (B) were left unstimulated or stimulated with either TNFα or anti-LTβR for the indicated times. Nuclear extracts were prepared and incubated with ^{32}P-labeled probes corresponding to the consensus κB site (NF-κB) or the *Blc*-κB and *Elc*-κB sites. DNA binding activity was analyzed by EMSA. NF-1 DNA binding activity was measured as an internal control. Panel (C) shows an exemplary functional analysis of the different κB sites in the *Blc* and *Elc* promoters. Triple repeats of the consensus κB (conκB), *Blc*-κB and a mutant *Blc*-κB (m*Blc*-κB) site were cloned upstream to a minimal SV40 promoter (pGL3-Promoter vector, Promega). In addition, the *Blc* (+12 to -688) and *Elc* (+530 to -320) promoter regions were cloned upstream to a luciferase reporter (pGL3-Basic vector, Promega). To determine the importance of the *Blc*-κB site, it was converted by site directed mutagenesis either to an inactive mutant version (mκB) or the consensus κB (conκB) site. The different plasmids were transfected into WT and *Ikkα*$^{-/-}$ MEFs. After 6 hrs with TNFα or anti-LTβR, luciferase activity was determined. The results are averages ± SD of three independent experiments normalized to β–galactosidase activity produced by a cotransfected β-galactosidase expression vector. Panel (D) shows an exemplary alignment of novel κB sites from the control regions of IKKα-dependent genes. The novel κB sites from the *Blc*, *Elc* and *Sdf-1* 5' regulatory region were aligned with those identified by computer analysis in the regulatory regions of three other IKKα-dependent genes. These sites form a consensus sequence (Alt. consensus) that although similar is distinct from the one associated with the classical NF-κB pathway (Class. consensus). Panel (E) shows an exemplary IKKα-dependent induction of *Baff*, *Rxra* and *Irf3*. Expression of the indicated

mRNAs was analyzed by Real Time-PCR as described above using RNA isolated from non-stimulated and anti-LTβR-stimulated stromal cells (*Rxra* and *Irf3*) and BMDCs (*Baff*) of the indicated genotypes.

Figure 31 shows an exemplary amino acid sequence (SEQ ID NO:130) of human NFκ-B2 (p52/p100) (GenBank accession number P23246).

Figure 32 shows an exemplary nucleotide sequence (SEQ ID NO:131) encoding human NFκ-B2 (p52/p100) (GenBank accession number BC051192).

Figure 33 shows an exemplary amino acid sequence (SEQ ID NO:132) of mouse NFκ-B2 (p52/p100) (GenBank accession number NP_076092).

Figure 34 shows an exemplary nucleotide sequence (SEQ ID NO:133) encoding mouse NFκ-B2 (p52/p100) (GenBank accession number NM_023603).

DEFINITIONS

To facilitate understanding of the invention, a number of terms are defined below.

The terms "purified," "to purify," "purification," "isolated," "to isolate," "isolation," and grammatical equivalents thereof as used herein, refer to the reduction in the amount of at least one contaminant from a sample. For example, a nucleotide sequence is purified by at least a 10%, preferably by at least 30%, more preferably by at least 50%, yet more preferably by at least 75%, and most preferably by at least 90%, reduction in the amount of undesirable proteins and/or undesirable nucleic acids, such as those present in a nuclear and/or cytoplasmic cell extract. Thus purification of a nucleotide sequence results in an "enrichment," *i.e.*, an increase in the amount, of the nucleotide sequence in the sample.

As used in this specification and the appended claims, the singular forms "a," "an" and "the" includes both singular and plural references unless the content clearly dictates otherwise.

As used herein, the term "or" when used in the expression "A or B," where A and B refer to a composition, disease, product, *etc.*, means one, or the other, or both.

The term "on" when in reference to the location of a first article with respect to a second article means that the first article is on top and/or into the second article, including, for example, where the first article permeates into the second article after initially being placed on it.

As used herein, the term "comprising" when placed before the recitation of steps in a method means that the method encompasses one or more steps that are additional to those

expressly recited, and that the additional one or more steps may be performed before, between, and/or after the recited steps. For example, a method comprising steps a, b, and c encompasses a method of steps a, b, x, and c, a method of steps a, b, c, and x, as well as a method of steps x, a, b, and c. Furthermore, the term "comprising" when placed before the recitation of steps in a method does not (although it may) require sequential performance of the listed steps, unless the content clearly dictates otherwise. For example, a method comprising steps a, b, and c encompasses, for example, a method of performing steps in the order of steps a, c, and b, the order of steps c, b, and a, and the order of steps c, a, and b, *etc.*

Unless otherwise indicated, all numbers expressing quantities of ingredients, properties such as molecular weight, reaction conditions, and so forth as used in the specification and claims are to be understood as being modified in all instances by the term "about." Accordingly, unless indicated to the contrary, the numerical parameters in the specification and claims are approximations that may vary depending upon the desired properties sought to be obtained by the present invention. At the very least, and without limiting the application of the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques. Notwithstanding that the numerical ranges and parameters describing the broad scope of the invention are approximation, the numerical values in the specific examples are reported as precisely as possible. Any numerical value, however, inherently contains standard deviations that necessarily result from the errors found in the numerical value's testing measurements.

The term "not" when preceding, and made in reference to, any particularly named molecule (such as RelA, RelB, mRNA, *etc.*) or phenomenon (such as biological activity, biochemical activity, *etc.*) means that the particularly named molecule or phenomenon is excluded, unless this term is defined differently. In particular, the term "does not bind to a protein" when made in reference to the binding of nucleotide sequence comprising the invention's RelBκB sequences (*e.g.*, SEQ ID NO:57) is a relative term that means that the level of binding of the nucleotide sequence comprising the invention's RelBκB sequences (*e.g.*, SEQ ID NO:57) to the protein is reduced by at least 5%, preferably at least 10%, more preferably at least 25%, yet more preferably at least 50%, further more preferably at least 75%, and most preferably at least 90%, relative to the level of binding of the nucleotide sequence comprising the invention's RelBκB sequences (*e.g.*, SEQ ID NO:57) to a polypeptide sequence comprising SEQ ID NO:62. The term "does not bind to a protein"

need not, although it may, mean an absolute absence of binding of the nucleotide sequence comprising the invention's RelBκB sequences (*e.g.*, SEQ ID NO:57) with the protein. The invention does not require, and is not limited to nucleotides sequences that 100% do not bind with the protein.

The term "altering" and grammatical equivalents as used herein in reference to the level of any a substance (*e.g.*, "RelB," "RelA," "p50," "RelB:50," "RelA:p50," "RelA:p52," "RelB RHD," "RelB DD," "Rel A RHD," and "Rel A DD," *etc.*) and/or phenomenon (*e.g.*, binding, expression, transcription, enzyme activity, pain, *etc.*) refers to an increase and/or decrease in the quantity of the substance and/or phenomenon, regardless of whether the quantity is determined objectively, and/or subjectively.

The term "increase," "elevate," "raise," and grammatical equivalents when in reference to the level of a substance (*e.g.*, "RelB," "RelA," "p50," "RelB:50," "RelA:p50," "RelA:p52," "RelB RHD," "RelB DD," "Rel A RHD," and "Rel A DD," *etc.*) and/or phenomenon (*e.g.*, binding, expression, transcription, enzyme activity, pain, *etc.*) in a first sample relative to a second sample, mean that the quantity of the substance and/or phenomenon in the first sample is higher than in the second sample by any amount that is statistically significant using any art-accepted statistical method of analysis. In one embodiment, the increase may be determined subjectively, for example when a patient refers to their subjective perception of disease symptoms, such as pain, clarity of vision, *etc.*. In another embodiment, the quantity of the substance and/or phenomenon in the first sample is at least 10% greater than the quantity of the same substance and/or phenomenon in a second sample. In another embodiment, the quantity of the substance and/or phenomenon in the first sample is at least 25% greater than the quantity of the same substance and/or phenomenon in a second sample. In yet another embodiment, the quantity of the substance and/or phenomenon in the first sample is at least 50% greater than the quantity of the same substance and/or phenomenon in a second sample. In a further embodiment, the quantity of the substance and/or phenomenon in the first sample is at least 75% greater than the quantity of the same substance and/or phenomenon in a second sample. In yet another embodiment, the quantity of the substance and/or phenomenon in the first sample is at least 90% greater than the quantity of the same substance and/or phenomenon in a second sample.

The terms "reduce," "inhibit," "diminish," "suppress," "decrease," and grammatical equivalents when in reference to the level of a substance (*e.g.*, "RelB," "RelA," "p50,"

"RelB:p50," "RelA:p50," "RelA:p52," "RelB RHD," "RelB DD," "Rel A RHD," and "Rel A DD," *etc.*) and/or phenomenon (*e.g.*, binding, expression, transcription, enzyme activity, pain, *etc.*) in a first sample relative to a second sample, mean that the quantity of substance and/or phenomenon in the first sample is lower than in the second sample by any amount that is statistically significant using any art-accepted statistical method of analysis. In one embodiment, the reduction may be determined subjectively, for example when a patient refers to their subjective perception of disease symptoms, such as pain, clarity of vision, *etc.*. In another embodiment, the quantity of substance and/or phenomenon in the first sample is at least 10% lower than the quantity of the same substance and/or phenomenon in a second sample. In another embodiment, the quantity of the substance and/or phenomenon in the first sample is at least 25% lower than the quantity of the same substance and/or phenomenon in a second sample. In yet another embodiment, the quantity of the substance and/or phenomenon in the first sample is at least 50% lower than the quantity of the same substance and/or phenomenon in a second sample. In a further embodiment, the quantity of the substance and/or phenomenon in the first sample is at least 75% lower than the quantity of the same substance and/or phenomenon in a second sample. In yet another embodiment, the quantity of the substance and/or phenomenon in the first sample is at least 90% lower than the quantity of the same substance and/or phenomenon in a second sample.

Reference herein to any specifically named protein (such as "RelB," "RelA," "p50," "RelB:p50," "RelA:p50," "RelA:p52," "RelB RHD," "RelB DD," "Rel A RHD," and "Rel A DD," *etc.*) refers to any and all equivalent fragments, fusion proteins, and variants of the specifically named protein having at least one of the biological activities (such as those disclosed herein and/or known in the art) of the specifically named protein, wherein the biological activity is detectably by any method. The term "fragment" when in reference to a protein refers to a portion of that protein that may range in size from four (4) contiguous amino acid residues to the entire amino acid sequence minus one amino acid residue. Thus, a polypeptide sequence comprising "at least a portion of an amino acid sequence" comprises from four (4) contiguous amino acid residues of the amino acid sequence to the entire amino acid sequence.

The term A "variant" of a protein as used herein is defined as an amino acid sequence which differs by insertion, deletion, and/or conservative substitution of one or more amino acids from the protein. The term "conservative substitution" of an amino acid refers to the replacement of that amino acid with another amino acid which has a similar

hydrophobicity, polarity, and/or structure. For example, the following aliphatic amino acids with neutral side chains may be conservatively substituted one for the other: glycine, alanine, valine, leucine, isoleucine, serine, and threonine. Aromatic amino acids with neutral side chains which may be conservatively substituted one for the other include phenylalanine, tyrosine, and tryptophan. Cysteine and methionine are sulphur-containing amino acids which may be conservatively substituted one for the other. Also, asparagine may be conservatively substituted for glutamine, and *vice versa*, since both amino acids are amides of dicarboxylic amino acids. In addition, aspartic acid (aspartate) my be conservatively substituted for glutamic acid (glutamate) as both are acidic, charged (hydrophilic) amino acids. Also, lysine, arginine, and histidine may be conservatively substituted one for the other since each is a basic, charged (hydrophilic) amino acid. Guidance in determining which and how many amino acid residues may be substituted, inserted or deleted without abolishing biological and/or immunological activity may be found using computer programs well known in the art, for example, DNAStar™ software. In one embodiment, the sequence of the variant has at least 95% identity with the sequence of the protein in issue. In another embodiment, the sequence of the variant has at least 90% identity with the sequence of the protein in issue. In yet another embodiment, the sequence of the variant has at least 85% identity with the sequence of the protein in issue. In a further embodiment, the sequence of the variant has at least 80% identity with the sequence of the protein in issue. In yet another embodiment, the sequence of the variant has at least 75% identity with the sequence of the protein in issue. In another embodiment, the sequence of the variant has at least 70% identity with the sequence of the protein in issue. In another embodiment, the sequence of the variant has at least 65% identity with the sequence of the protein in issue.

Reference herein to any specifically named nucleotide sequence (such as 5'-NGGAGANNTG-3' (SEQ ID NO:57), *etc.*) includes within its scope any and all equivalent fragments, homologs, and sequences that hybridize under high and/or medium stringent conditions to the specifically named nucleotide sequence, and that have at least one of the biological activities (such as those disclosed herein and/or known in the art) of the specifically named nucleotide sequence, wherein the biological activity is detectably by any method. The "fragment" may range in size from an exemplary 6, 7, 8, and 9, contiguous nucleotide residues to the entire nucleic acid sequence minus one nucleic acid residue. Thus, a nucleic acid sequence comprising "at least a portion of" a nucleotide sequence

comprises from six (6) contiguous nucleotide residues of the nucleotide sequence to the entire nucleotide sequence.

The term "homolog" of a specifically named nucleotide sequence refers to an oligonucleotide sequence which exhibits greater than or equal to 50% identity to the specifically named nucleotide sequence when sequences having a length of 8 bp or larger are compared. Alternatively, a homolog of a specifically named nucleotide sequence is defined as an oligonucleotide sequence which has at least 95%, at least 90%, at least 85%, at least 75%, at least 70%, or at least 65%, identity with the specifically named nucleotide sequence in issue.

With respect to sequences that hybridize under stringent condition to the specifically named nucleotide sequence, high stringency conditions comprise conditions equivalent to binding or hybridization at 68°C in a solution containing 5X SSPE, 1% SDS, 5X Denhardt's reagent and 100 μg/ml denatured salmon sperm DNA followed by washing in a solution containing 0.1X SSPE, and 0.1% SDS at 68°C when a probe of about 100 to about 1000 nucleotides in length is employed. "Medium stringency conditions" when used in reference to nucleic acid hybridization comprise conditions equivalent to binding or hybridization at 42°C in a solution of 5X SSPE (43.8 g/l NaCl, 6.9 g/l NaH2PO4-H2O and 1.85 g/l EDTA, pH adjusted to 7.4 with NaOH), 0.5% SDS, 5X Denhardt's reagent and 100 μg/ml denatured salmon sperm DNA followed by washing in a solution comprising 1.0X SSPE, 1.0% SDS at 42°C when a probe of about 500 nucleotides in length is employed.

The term "equivalent" when made in reference to a hybridization condition as it relates to a hybridization condition of interest means that the hybridization condition and the hybridization condition of interest result in hybridization of nucleic acid sequences which have the same range of percent (%) homology. For example, if a hybridization condition of interest results in hybridization of a first nucleic acid sequence with other nucleic acid sequences that have from 85% to 95% homology to the first nucleic acid sequence, then another hybridization condition is said to be equivalent to the hybridization condition of interest if this other hybridization condition also results in hybridization of the first nucleic acid sequence with the other nucleic acid sequences that have from 85% to 95% homology to the first nucleic acid sequence.

As will be understood by those of skill in the art, it may be advantageous to produce a nucleotide sequence encoding a protein of interest, wherein the nucleotide sequence possesses non-naturally occurring codons. Therefore, in some preferred embodiments,

codons preferred by a particular prokaryotic or eukaryotic host (Murray *et al.*, Nucl. Acids Res., 17 (1989)) are selected, for example, to increase the rate of expression or to produce recombinant RNA transcripts having desirable properties, such as a longer half-life, than transcripts produced from naturally occurring sequence.

The term "naturally occurring" as used herein when applied to an object (such as cell, *etc.*) and/or chemical (such as amino acid, amino acid sequence, nucleic acid, nucleic acid sequence, codon, *etc.*) means that the object and/or compound can be found in nature. For example, a naturally occurring polypeptide sequence refers to a polypeptide sequence that is present in an organism (including viruses) that can be isolated from a source in nature, wherein the polypeptide sequence has not been intentionally modified by man in the laboratory.

The term "chosen from A, B and C" means selecting one or more of A, B, and C.

A "composition comprising a particular nucleotide sequence" as used herein refers broadly to any composition containing the recited nucleotide sequence. The composition may comprise an aqueous solution containing, for example, salts (*e.g.*, NaCl), detergents (*e.g.*, SDS), and other components (*e.g.*, Denhardt's solution, dry milk, salmon sperm DNA, *etc.*).

DESCRIPTION OF THE INVENTION

The invention provides nucleotide sequences that mediate one or more functions of IKKα, kits and methods for using these sequences to identify therapeutic compounds that alter IKKα related pathology. The present invention relates to compositions and methods of altering signal transduction of extracellular signals that is mediated by IKKα into specific patterns of gene expression and, thus, of altering IKKα-mediated gene expression in the cells in which it occurs.

IκB Kinase (IKK)α is required for activation of an alternative NF-κB signaling pathway based on processing of the NF-κB2/p100 precursor protein, which associates with RelB in the cytoplasm. This pathway, which leads to activation of RelB:p52 dimers, is required for induction of several chemokine genes needed for organization of secondary lymphoid organs. We investigated why induction of these genes in response to engagement of the lymphotoxin β receptor (LTβR) is selectively dependent on the alternative NF-κB signaling pathway. Using chromatin immunoprecipitation, data herein shows that the promoters of these exemplary genes are recognized by RelB:p52 dimers and not RelA:p50

dimers, the ubiquitous target for the classical NF-κB signaling pathway. Furthermore, data herein demonstrates the identification in these exemplary promoters of a novel type of NF-κB binding site that is preferentially recognized by RelB:p52 dimers. This site links induction of organogenic chemokines to activation of the alternative pathway.

Two distinct pathways leading to selective activation of RelA:p50 and RelB:p52 dimers, dependent on IKKβ or IKKα, respectively, were identified (S. Ghosh, M. Karin, *Cell* 109, S81-96 (2002)). Each pathway has distinct biological functions (Q. Li, D. Van Antwerp, F. Mercurio, K.-F. Lee, I. M. Verma, *Science* 284, 321-325 (1999); L.-W. Chen *et al.*, *Nat Med* 9, 575-581 (2003); U. Senftleben *et al.*, *Science* 293, 1495-1499 (2001)), that could be mediated in part through selective gene activation (E. Dejardin *et al.*, *Immunity* 17, 525-535 (2002)). How this occurs was previously unknown. Data herein shows in two different cell types, splenic stromal cells and BMDC, that IKKα is required for induction of four genes encoding chemokines critical for organogenesis of the spleen and maintenance of its microarchitecture because these genes are selectively recognized by RelB-containing dimers, most likely RelB:p52 (Figure 6). These genes are preferentially activated by engagement of LTβR and are weakly responsive to TNFα. Whereas the induction by TNFα of these and other genes is IKKα-independent, the response to LTβR engagement is strictly IKKα-dependent, because of two events. First, RelB:p52 dimers have to enter the nucleus, a process dependent on IKKα-mediated p100 processing (E. Dejardin *et al.*, *Immunity* 17, 525-535 (2002); Z. B. Yilmaz, D. S. Weih, V. Sivakumar, F. Weih, *Embo J* 22, 121-130 (2003)). Second, RelB:p52 dimers are selectively recruited to the IKKα-dependent gene promoter. The selective recruitment of RelB to the *Blc* and the *Elc* promoters is likely to depend on a novel κB site that is much more effectively recognized by RelB:p52 in comparison to RelA:p50 or even RelB:p50. It is the inventors' consideration that this unique specificity of RelB:p52 dimers is entirely consistent with sequence differences between the DNA binding loops of RelA and RelB, but was previously unknown (G. Ghosh, G. Vanduyne, S. Ghosh, P. B. Sigler, *Nature* 373, 303-310 (1995)). The inventors also consider that additional factors may contribute to selective IKKα-dependent gene activation and that IKKα may also be responsible in certain cell types for activation of the canonical NF-κB pathway (Y. Cao *et al.*, *Cell* 107, 763-775 (2001)) or for potentiating its ability to activate transcription (A. Israel, *Nature* 423, 596-597 (2003); Y. Yamamoto *et al.*, *Nature* 423, 655-659 (2003); V. Anest *et al.*, *Nature* 423, 659-63 (Jun 5, 2003)).

Nonetheless, the major mechanism responsible for selective gene activation through the IKKα-dependent alternative NF-κB signaling pathway is based on specific recruitment of RelB:p52 dimers to target gene promoters.

Without intending to limit the invention to any particular mechanism, and while an understanding of mechanism is not required, it is the inventors' consideration that the functional separation between the two NF-κB signaling pathways optimizes adaptive immunity through proper organization of secondary lymphoid organs. By contrast, IKKβ is mostly involved in inflammatory and innate immune responses. Thus IKKβ-mediated NF-κB signaling is responsible for rapid responses to infection and injury, that require recruitment of immune cells out of lymphoid organs to sites of infection. This response depends on pro-inflammatory chemokines, such as MIP-1, MCP-1 and RANTES, which are induced by the canonical NF-κB signaling pathway (E. Alcamo *et al., J Immunol* 167, 1592-1600 (2001)) (Hoffman, A. *et al.*, personal communication). The arrival of antigens to secondary lymphoid tissues from distal sites of infection and its processing, presentation and recognition require coordinated activity of DC, macrophages, T cells and B cells, whose recruitment to secondary lymphoid organs depends on IKKα-regulated organogenic chemokines. Premature expression of such chemokines would compromise the immediate anti-microbial response as it may abort the emigration of immune cells to the periphery. It is, therefore, the inventors' view that the genes for organogenic chemokines are not activated by the canonical NF-κB signaling pathway. Consistent with its delayed function in adaptive immunity, activation of the alternative, IKKα-dependent, NF-κB signaling pathway is slower than activation of the canonical NF-κB signaling pathway and may depend on prior activation of the latter (E. Dejardin *et al.*, *Immunity* 17, 525-535 (2002)). The dependence of the two pathways on distinct but related protein kinases and transcription factors allows for both functional integration and kinetic separation.

To facilitate understanding of the invention, the invention is further described under (A) The NFκB signalling pathways, (B) IKKα and the generation of biological specificity, (C) The biological functions of IKKα and RelB:p52, (D) Target DNA sequences for NFκ-B2/p52, (E) Methods for identifying test compounds that alter RelB DNA-binding activity, (F) Detection of specific binding, and screening test compounds, using arrays, (G) Detection of specific binding, and screening test compounds, using electrophoretic mobility shift (EMS) assays, (H) Detection of specific binding, and screening test compounds, using

footprinting Assays, (I) Detection of specific binding, and screening test compounds, using reporter gene assays, (J) Detection of specific binding, and screening test compounds, using optical affinity biosensor system assays, (K) Methods for expressing a nucleic acid sequence of interest, (L) Methods of altering symptoms of diseases associated with IKKα pathology, (M) Kits, and (O) Additional considerations.

A. The NFκB signalling pathways

The mechanisms responsible for selective gene activation by closely related transcription factors, which lie downstream to different members of the same cell surface receptor family, remain largely unsolved and represent a major problem in the molecular biology of signal transduction. The NF-κB family of transcription factors represents one such case. This family consists of five subunits: RelA, c-Rel, RelB, NF-κB1/p50 and NF-κB2/p52, that form more than a dozen dimers (Ghosh *et al.* (1998) Ann Rev Immunol 16, 225-260). *In vitro* these dimers appear to recognize DNA binding sites, including a κB site (Huxford *et al.* (1999) Cold Spring Harb Symp Quant Biol 64, 533-540; Attar *et al.* (1997) Semin Cancer Biol 8, 93-101; Gerondakis *et al.* (1999) Oncogene 18, 6888-6895; Sanjabi *et al.* (2000) Proc Natl Acad Sci U S A 97, 12705-12710). How each NF-κB dimer selectively activates distinct set of genes is not clear and it seems to be a general problem applicable to other families of closely related transcription factors that serve as nuclear targets for signals emanating at cell surface receptors (Massague *et al.* (2000) Cell 103, 295-309).

One step towards explanation of biological specificity in the NF-κB response entailed the description of two distinct NF-κB signaling pathways. The canonical NF-κB signaling pathway, which is activated by proinflammatory cytokines and pathogen associated molecular patterns (PAMPs), depends on inducible degradation of specific inhibitors, IκBs, which retain most NF-κB dimers in the cytoplasm (reviewed by Ghosh and Karin, 2002; Ghosh *et al.* (2002) Cell, 109, S81-96). This pathway is largely dependent on IKKβ, a catalytic subunit of a complex that also contains the IKKαcatalytic subunit and the IKKγ/NEMO regulatory subunit (reviewed by Rothwarf and Karin, 1999; Ghosh *et al.* (1998) Ann Rev Immunol, 16, 225-260). In this pathway, IKKβ phosphorylates IκBs at N-terminal sites to trigger their ubiquitin-dependent degradation and subsequent nuclear entry of NF-κB dimers, which mostly contain RelA and c-Rel, as the transcription activating

subunits (reviewed by Ghosh and Karin, 2002; Karin *et al.* (2000) Annu Rev Immunol, 18, 621-663).

A second, alternative NF-κB activation pathway based on regulated processing of the NF-κB2/p100 precursor protein was also described (Senftleben *et al.* (2001a) Science 293, 1495-1499; Xiao *et al.* (2001) Mol Cell 7, 401-409). NF-κB2/p100 contains an N-terminal Rel homology domain (RHD), common to all NF-κB proteins, and an inhibitory IκB-like C-terminal domain (Ghosh *et al.* (1998) Ann Rev Immunol 16, 225-260). The presence of the latter prevents nuclear translocation of p100 and its partners. The preferred cytoplasmic partner for p100 is RelB (Solan *et al.* (2002) J Biol Chem 277, 1405-1418). Overexpression of the protein kinase NIK causes ubiquitin-dependent degradation of the p100 C-terminal domain and release of mature NF-κB2/p52, which contains the N-terminal RHD (Xiao *et al.* (2001) Mol Cell 7, 401-409).. This process depends on IKKα, which directly phosphorylates p100 in vitro at sites required for its processing, but does not require IKKβ (Senftleben *et al.* (2001a) Science 293, 1495-1499) or IKKγ (Claudio *et al.* (2002) Nat Immunol 3, 958-965; Yilmaz *et al.* (2003) EMBO J 22, 121-130) Dejardin *et al.* (2002) Immunity 17, 525-535). NF-κB2/p100 processing promotes the nuclear translocation of RelB (Dejardin *et al.* (2002) Immunity 17, 525-535; Solan *et al.* (2002) J Biol Chem 277, 1405-1418). Recently, this pathway was found to be selectively activated by certain members of the TNF cytokine family, including lymphotoxin (LT) $\alpha_1:\beta_2$ trimers acting through LTβ receptor (LTβR), B cell activating factor (BAFF; also known as Blys/TALL-1/THANK and B cell activating factor belonging to the TNF family) acting through BAFF-R and CD40 ligand (CD40L) whose receptor is CD40 (Claudio *et al.* (2002) Nat Immunol 3, 958-965; Cooper *et al.* (2002) EMBO J 21, 5375-5385; Dejardin *et al.* (2002) Immunity 17, 525-535; Kayagaki *et al.* (2002) Immunity 17, 515-524; Yilmaz *et al.* (2003) EMBO J 22, 121-130).

IKKα and IKKβ, activate at least a dozen NF-κB dimers, composed of five subunits (S. Ghosh, M. Karin, (2002) Cell, 109, S81-96). While the mechanisms of NF-κB activation are well understood (S. Ghosh, M. Karin, (2002) Cell, 109, S81-96), the generation of biological specificity by this complex system is more enigmatic (J.L. Pomerantz, D. Baltimore, *Mol Cell* 10, 693-695 (2002)). Mouse mutagenesis experiments indicate that IKKβ activates the classical NF-κB pathway, represented by RelA:p50 dimers, in response to stimuli such as tumor necrosis factor (TNF)α (Li *et al.* (1999) Science, 284,

321-325 and Chen *et al.* (2003) Nat Med, 9, 575-581). The mechanisms by which IKKα regulates cytokine-induced gene expression are more obscure and controversial (Israel *et al.* (2003) Nature, 423, 596-597). *In vivo* analysis revealed that IKKα activates an alternative NF-κB pathway based on processing of NF-κB2/p100 and release of RelB:p52 dimers (U. Senftleben *et al.*, *Science* 293, 1495-1499 (2001)) in response to LT α/β trimers (Dejardin *et al.* (2002) Immunity, 17, 525-535) and other TNF family members (Claudio *et al.* (2002) Nat Immunol, 3, 958-965; Kayagaki *et al.* (2002) Immunity, 17, 515-524). This pathway is involved in secondary lymphoid organogenesis and induction of genes involved in this process, but has no apparent role in TNFα-induced functions (Dejardin *et al.* (2002) Immunity, 17, 525-535; Senftleben *et al.* (2001) Science, 293, 1495-1499). We used mice in which IKKα was rendered inactivateable (Cao *et al.* (2001) Cell, 107, 763-775) to study the mechanism responsible for selective gene induction by the alternative NF-κB signaling pathway. Using primary cultures of splenic stromal cells, myeloid dendritic cells, and bone marrow-derived myeloid dendritic cells (BMDCs) we found that generation of gene induction specificity by IKKα depends on selective activation of RelB:p52 dimers, which recognize a unique type of NF-κB binding sites. This novel cis element is responsible for rendering the induction of chemokines involved in secondary lymphoid organogenesis IKKα- dependent.

IKKα plays a role in proper compartmentalization of the spleen, germinal center (GC) formation and development of other secondary lymphoid organs (Kaisho *et al.* (2001) J Exp Med 193, 417-426; Senftleben *et al.* (2001a) Science 293, 1495-1499). Lethally irradiated mice reconstituted with IKKα-deficient hematopoietic stem cells lack GCs and marginal zone macrophages and exhibit reduced B cell maturation, suggesting that IKKα acts within hematopoietic derivatives (Kaisho *et al.* (2001) J Exp Med 193, 417-426; Senftleben *et al.* (2001a) Science 293, 1495-1499). Indeed, loss of IKKα activity interferes with p100 processing in B cells, without affecting activation of the canonical NF-κB pathway (Senftleben *et al.* (2001a) Science 293, 1495-1499). As B cells play a role in inducing mature follicular dendritic cell (FDC) networks and the latter in turn play a role in GC formation (Cyster *et al.* (2000) Immunol Rev 176, 181-193; Fu *et al.* (1999) Annu Rev Immunol 17, 399-433), it is not clear whether the defects in spleen development and organization are secondary to the B cell autonomous function of IKKα or reflect a role for IKKα in other compartments. Similar defects are also displayed by *Nfkb2*$^{-/-}$ and *RelB*$^{-/-}$

mice. Furthermore, whereas two of the receptors whose activation leads to p100 processing, BAFF-R and CD40, are expressed on B cells, the third receptor, LTβR, is expressed on stromal cells of secondary lymphoid organs (Fu *et al.* (1999) Annu Rev Immunol 17, 399-433). LTβR signaling is involved in stromal cell function and *Ltbr*$^{-/-}$ mice exhibit defective development of secondary lymphoid organs (Fu *et al.* (1999) Annu Rev Immunol 17, 399-433).

Injection of agonistic anti-LTβR antibody into wild type (WT) and *Ikkα*$^{AA/AA}$ mice, which express a non-activatable form of IKKα (Cao *et al.* (2001) Cell 107, 763-775), revealed interesting changes in gene expression within the spleen. Certain genes, such as those encoding chemokines SLC/CCL21, ELC/CCL19, BLC/CXCL13 and SDF-1/CXCL12, were induced in WT but not the *Ikkα*$^{AA/AA}$ spleen, whereas other genes, such as *Vcam-1, Mip-1β* and *Mip-2*, were more efficiently induced in the *Ikkα*$^{AA/AA}$ spleen (Dejardin *et al.* (2002) Immunity 17, 525-535). Although these differences in gene expression were correlated with defective LTβR-induced p100 processing, the molecular basis for this gene induction pattern was not determined. Moreover the target cell type(s) in which these genes are differentially expressed have not been identified. This is of importance since the chemokines whose induction is IKKα-dependent control many of the critical cell-cell interactions and cell migration patterns involved in morphogenesis of the spleen and other secondary lymphoid organs (Ansel *et al.* (2001) Curr Opin Immunol 13, 172-179; Kim *et al.* (1999) J Leukoc Biol 65, 6-15).

The inventors further investigated the mechanisms responsible for the IKKα-dependent induction of the *Slc, Elc, Blc*, and *Sdf-1* genes, as they offer an excellent model system for understanding the basis for the different biological functions and target gene specificity of the two NF-κB activation pathways (Pomerantz *et al.* (2002) Mol Cell 10, 693-695). The inventors first identified the exemplary cells in which IKKα activity plays a role in induction of these genes as the stromal cells of the spleen, where they are directly induced in response to LTβR engagement. Next, the inventors found that at least two of the four chemokine gene promoters are directly recognized by RelB-containing, but not by RelA-containing, NF-κB dimers. Recruitment of RelB to these promoters correlates with their transcriptional activation. Furthermore, at least one of these promoters contains a novel κB site that is selectively recognized by RelB:p52 dimers. While an understanding of the mechanism of the invention is not necessary, and without limiting the invention to any

particular mechanism, data herein shows that the IKKα provides receptor selectivity for the RelB-dependent response; in this case it allows its activation by LTβR but not by the related type 1 TNFα receptor (TNFR1). Further, data herein shows that IKKα provides RelB with the proper heterodimeric partner, which is p52 rather than p50. Nuclear translocation of RelB in the absence of p52, brought about by TNFα is not sufficient for rapid activation of the IKKα-dependent chemokine genes in stromal cells. Thus, in cells where its amounts are limiting, RelB activates specific target genes when complexed with p52.

B. IKKα and the generation of biological specificity

The NF-κB family consists of five subunits, forming more than a dozen different dimers. Each family member may activate distinct target genes in addition to a common gene set. How this specificity and diversity is established has not, heretofore, been clear. While an understanding of the mechanism of the invention is not necessary, and without intending to limit the invention to any mechanism, it is the inventors view that IκB kinase α (IKKα) activates an alternative NF-κB pathway by inducing processing of NF-κB2/p100 and release of RelB:p52 dimers. This pathway is selectively activated in splenic stromal cells after lymphotoxin β receptor engagement causing activation of specific genes, required for maintenance of splenic microarchitecture. At least two of these genes are selectively recognized in vivo by RelB-containing dimers but not by RelA-containing dimers. Data herein shows that IKKα provides the specificity for this response both by allowing its selective activation by certain cell surface receptors and not others, and by providing RelB with the proper heterodimeric partner, p52, leading to recognition of unique DNA sites.

It is the inventors' view that two distinct pathways lead to activation of NF-κB transcription factors. The canonical pathway depends on the IKKβ catalytic subunit of the IKK complex which targets IκB proteins to degradation. This pathway activates classical NF-κB dimers, the most common of which is the RelA:p50 heterodimer (Ghosh *et al.* (2002) Cell 109, S81-96). The second pathway, termed the alternative pathway, depends on the IKKα catalytic subunit, acting in isolation from IKKβ or IKKγ/NEMO, IKKα targets the NF-κB2/p100 precursor protein to proteolytic processing, leading to activation of RelB:p52 dimers (Ghosh *et al.* (2002) Cell 109, S81-96). Mutational analysis in mice indicated that the two pathways have distinct biological functions (Li *et al.* (1999a) Science

284, 321-325; Li *et al.* (1999b) J Exp Med 189, 1839-1845; Senftleben *et al.* (2001a) Science 293, 1495-1499; Senftleben *et al.* (2001b) Immunity 14, 217-230) and even provided preliminary evidence that they may activate distinct target genes (Dejardin *et al.* (2002) Immunity 17, 525-535). The ability of both pathways to be activated within the same cell in response to engagement of a single receptor, for instance the LTβR, each leading to induction of a distinct gene set has raised questions regarding the mechanism responsible for target gene specificity (Pomerantz *et al.* (2002) Mol Cell 10, 693-695). In fact, how activation of structurally related transcription factors within the same cell results in activation of distinct target genes leading to different biological responses, is a general question that applies to many signal transduction pathways involved in gene regulation. Data herein shows the ability of the alternative NF-κB signaling pathway to selectively activate a set of target genes that are not responsive to the canonical pathway.

Data herein shows that IKKα provides target gene specificity at two levels. First, it allows RelB:p52 dimers to be activated in response to engagement of certain members of the TNFR family. As shown here, RelB and p52 are simultaneously present in nuclei of fibroblasts or stromal cells after engagement of LTβR but not after occupancy of TNFR1. Second, IKKα provides RelB with the proper heterodimeric partner: p52 instead of p50. As shown above, RelB:p52 dimers interact more effectively with the *Blc* κB site than RelB:p50 dimers. Nevertheless, this selectivity, as discussed below, is not absolute. The second function was surprising as it was thought that RelB nuclear translocation is tightly linked to processing of p100, with which RelB associates in the cytoplasm (Dejardin *et al.* (2002) Immunity 17, 525-535; Solan *et al.* (2002) J Biol Chem 277, 1405-1418). Furthermore, it was believed that RelB:p52 dimers are not different in their sequence selectivity from RelA:p50 or RelB:p50 dimers. Thus the sole role of IKKα was thought to be induction of RelB nuclear entry via processing of RelB:p100 dimers. We found, however, that stimulation of either MEFs or splenic stromal cells with TNFα results in effective nuclear translocation of RelB in the absence of p100 processing. Nevertheless, TNFα does not lead to as efficient recruitment of RelB-containing dimers to the IKKα-dependent target genes as anti-LTβR does. The delayed and weak recruitment of RelB to the *Blc* and *Sdf-1* promoters (exemplary sequence in Figure 7) after TNFα stimulation is likely to be due to the formation of RelB:p50 dimers, whose concentration may be sufficiently high several hrs after TNFR1 engagement. By contrast, the more rapid and robust recruitment of RelB to

the same promoters seen after stimulation of LTβR is likely to be due to formation of RelB:p52 dimers. As RelB:p52 dimers bind more effectively to the type of κB site present in the *Blc* promoter, their effective concentration does not need to be as high as that of concentration of RelB:p50 dimers.

These conclusions are derived from several different experiments. First, NF-κB:DNA complexes formed by incubation of a canonical κB site with nuclear extracts can be supershifted by anti-RelB antibodies when the nuclear extracts are from LTβR-stimulated and not from TNFα-stimulated cells. Second, ChIP experiments reveal that RelB is recruited to target gene promoters more rapidly and efficiently in anti-LTβR-stimulated than in TNFα-stimulated cells. Third, RelB:p52 dimers bind much more efficiently to the *Blc* κB site than RelB:p50 and especially RelA:p50 dimers. In addition, the conclusion that selective activation of the *Blc, Sdf-1, Elc and Slc* genes is dependent on RelB:p52 dimers is strongly supported by the results of two other gene disruption experiments, as expression of these genes is defective in spleens of $RelB^{-/-}$ and $Nf\kappa b2^{-/-}$ mice but not in $Nf\kappa b1^{-/-}$ mice (Weih *et al.* (2001) J Immunol 167, 1909-1919). Like the $Ikk\alpha^{AA}$ mutation, the *RelB* and *Nfκb2* gene disruptions have no effect on the expression of TNFα or chemokine receptors.

While an understanding of the mechanism of the invention is not necessary, and without intending to limit the invention to any particular mechanism, it is the inventors' view that RelB appears to differ in its DNA binding properties from the two other Rel proteins: RelA and c-Rel. Whereas RelA and c-Rel can bind DNA as homodimers, RelB homodimers have very poor DNA binding activity. The binding of RelB to DNA is therefore dependent on heterodimerization with p50 or p52. Whereas RelB:p52 and RelB:p50 dimers bind with similar efficiency to the consensus κB site, the *Blc* κB site is more efficiently recognized by RelB:p52 dimers. Furthermore, the *Blc* κB site is poorly recognized by RelA:p50 dimers. The basis for DNA target site selection by different NF-κB dimers is not well understood, as RelA and p50 homo- and hetero-dimers have been studied (Phelps *et al.*, 2000; Chen *et al.* (1998) Nat Struct Biol 5, 67-73; Huang *et al.* (2001) Structure (Camb) 9, 669-678). While an understanding of the mechanism of the invention is not necessary, and without intending to limit the invention to any particular mechanism, it is the inventors' view that RelA and c-Rel discriminate against DNA sequences with a third G:C basepair. The inventor's view is that the classical RelA:p50 binding site is: 5'-

GGGRNWTTCC-3' (SEQ ID NO:120) (where R, N and W denote purine, any nucleotide and A or T, respectively). In this site, the 5' half site (5'-GGGRN-3') (SEQ ID NO:121) is occupied by p50 and the 3' half site (5'-TTCC-3') (SEQ ID NO:122) is occupied by RelA.

While an understanding of the mechanism of the invention is not necessary, and without intending to limit the invention to any particular mechanism, it is the inventors' view that the 5' half site (5'-GGGAG-3') (SEQ ID NO:66) of the *Blc* κB site, whose sequence is: 5'-GGGAGATTTG-3' (SEQ ID NO:59), is recognized by p52, while the 3' half site (5'-TTTG-3') (SEQ ID NO:67) is recognized by RelB. Also without limiting the invention to any particular mechanism, it is the inventors' view that the last two basepairs of the 3' half site interfere with its recognition by RelA. The inventors noted that RelB differs from RelA in the sequence of its DNA binding loops 1 and 3 (Ghosh *et al.* (1995) Nature 373, 303-310). For instance, RelB has a glutamic acid in loop 1 instead of a conserved lysine in RelA and c-Rel, and lysine and threonine in loop 3 instead of asparigine and proline in RelA. While an understanding of the mechanism of the invention is not necessary, and without intending to limit the invention to any particular mechanism, it is the inventors' view that it may be possible that these differences may be sufficient to alter the sequence selectivity of RelB from that of RelA. Furthermore, the inventors noted the differential DNA binding of RelA:p50 and RelA:p52 dimers (Nijnik *et al.* (2003) Nucleic Acids Res 31, 1497-1501; Schmid *et al.* (1994) J Biol Chem 269, 32162-32167). It is also the inventors' view that certain NF-κB sites, such as the consensus κB site, may be effectively recognized by all or most dimers, whereas other sites, such as the invention's RelBκB site, may be preferentially recognized by one type of dimer. Intriguingly, a very similar sequence to the invention's RelBκB site in *Blc* is present in the *Sdf-1* promoter.

C. The biological functions of IKKα and RelB:p52

The inventors observed that the RelB:p52-specific target genes whose activation depends on IKKα are functionally related, encoding major chemokines that play a role in the development and organization of the spleen and secondary lymphoid organs (Ansel *et al.* (2001) Curr Opin Immunol 13, 172-179; Kim *et al.* (1999) J Leukoc Biol 65, 6-15). Based on the functional analysis described above, IKKα also plays a role in for differentiation of FDCs, which most likely arise from stromal progenitors (Mackay *et al.* (1998) Nature 395, 26-27). Formation and maintenance of the mature FDC network is an active process that requires production of $LT\alpha_1\beta_2$ heterotrimers by B cells, which engage

LTβR on stromal cells (Mackay *et al.* (1998) Nature 395, 26-27). Although TNFR1 may also be involved in this process, its contribution is minor relative to that of LTβR (Mackay *et al.* (1998) Nature 395, 26-27). Indeed, the inventors found that TNFα fails to induce effective NF-κB2/p100 processing or expression of the four IKKα-dependent chemokine genes. We also found that injection of agonistic anti-TNFR1 antibody into $Ikk\alpha^{AA/AA}$ mice failed to promote FDC maturation.

The transplantation experiments described herein indicate that one of the major sites of IKKα action is the stromal cell, the cell type that expresses LTβR (Fu *et al.* (1999) Annu Rev Immunol 17, 399-433). Furthermore, the manifestations of disrupted LTβR signaling (Fu *et al.* (1999) Annu Rev Immunol 17, 399-433) are strikingly similar to those of the $Ikk\alpha^{AA}$ mutation. Thus, although IKKα is involved in NF-κB2/p100 processing in B cells and has a B cell autonomous function (Senftleben *et al.* (2001a) Science 293, 1495-1499), it is the inventors' view that most of the defects in spleen development and morphogenesis in $Ikk\alpha^{AA/AA}$ mice are probably due to defective LTβR signaling. Despite the similarities between the LTβR and IKKα deficiencies, engagement of LTβR also results in activation of the canonical NF-κB signaling pathway, leading to induction of genes such as *Vcam-1*, *Tnfα* and *Baff*, which are not dependent on IKKα. Without limiting the invention to any mechanism, these findings suggest to the inventors that either the canonical NF-κB signaling pathway is not required for the developmental and morphogenetic functions of LTβR, or that in the absence of LTβR, functions that depend on the canonical pathway can be induced by other receptors, for instance TNFR1. Interestingly, mice deficient in NF-κB2 or RelB expression exhibit splenic phenotypes that are quite similar to the one exhibited by $Ikk\alpha^{AA/AA}$ mice (Caamano *et al.* (1998) J Exp Med 187, 185-196; Franzoso *et al.* (1998) J Exp Med 187, 147-159; Poljak *et al.* (1999) J Immunol 163, 6581-6588; Weih *et al.* (2001) J Immunol 167, 1909-1919). Transplantation experiments revealed that the stromal cell is also a major site of NF-κB2 and RelB action (Franzoso *et al.* (1998) J Exp Med 187, 147-159; Weih *et al.* (2001) J Immunol 167, 1909-1919).

While an understanding of the mechanism of the invention is not necessary, and without limiting the invention to any particular mechanism, the inventors are of the view that IKKα is responsible for selective activation of RelB:p52 target genes whose major functions are in development and organization of secondary lymphoid organs as structures that optimize adaptive immunity, especially the T-cell dependent humoral response. IKKβ,

on the other hand, is mostly involved in inflammatory and innate immune responses. Thus IKKβ-mediated NF-κB signaling is in charge of rapid responses to infection and injury. At the early stage of the response to bacterial or viral pathogens, it is important to attract inflammatory cells (macrophages, neutrophils), as well as mature lymphocytes with the proper specificity out of lymphoid organs to sites of infection and injury. This targeting is dependent on pro-inflammatory chemokines, such as MIP-1, MCP-1 and RANTES, whose induction is dependent on activation of the canonical NF-κB signaling pathway. On the other hand, antigen arrives in the secondary lymphoid tissues from distal sites of infection and is recognized, processed and presented by the combined activity of DC, macrophages, T cells and B cells. Orchestration of these key cell-cell interactions is controlled by chemokines induced by the alternative NF-κB signaling pathway. Premature expression of such chemokines would compromise the response to infectious agents as it will cause the recruitment of myeloid and lymphoid cells from sites of infection back to lymphoid organs. It is, therefore the inventors' view that expression of chemokines involved in innate immune responses appears to be highly dependent on RelA (Alcamo *et al.* (2001) J Immunol 167, 1592-1600). At late stages of the response to infection, it becomes important to engage the adaptive immune response. Consistent with its delayed function, activation of the alternative, IKKα-dependent, NF-κB signaling pathway is slower than activation of the canonical NF-κB signaling pathway. The dependence of the two pathways on distinct but related protein kinases allows for both functional integration and kinetic separation. For instance, activation of IKKβ and the canonical pathway by pathogen associated molecular patterns (PAMPs), such as lipopolysaccharides (LPS), can lead to increased expression of cytokines, such as LTα, that activate the alternative pathway, as well as NF-κB2/p100 (Dejardin *et al.* (2002) Immunity 17, 525-535), which serves as a substrate for the IKKα-dependent processing pathway. The processing of p100 provides RelB with its partner, p52, leading to activation of genes that are involved in, for example, mounting the humoral response.

D. Target DNA sequences for NFκ-B2/p52

The invention provides a RelBκB nucleotide sequences that specifically bind to proteins, which are involved in the IKKα rather than in the IKKβ pathway. In particular, the invention provides an isolated nucleotide sequence comprising 5'-NGGAGANNTG-3'

(SEQ ID NO:57); wherein N at position 1 is chosen from G and A, N at position 7 is chosen from T and C, and N at position 8 is chosen from T and C (see, Figure 4C), and wherein the isolated sequence specifically binds with a polypeptide sequence comprising RelB Rel homology domain (RHD), which is exemplified by SEQ ID NO:62, *i.e.*, amino acids 1-400 of the exemplary mouse RelB shown in Figure 13, GenBank accession A42023. As used herein the terms "κB site," "κB sequence," "RelBκB site," "RelBκB sequence," and "RelBκB recognition sequence," "RelB-specific binding site," and "RelB-specific binding sequence" are used interchangeably.

The terms nucleotide sequence "comprising a particular nucleic acid sequence" and protein "comprising a particular amino acid sequence" and equivalents of these terms, refer to any nucleotide sequence of interest and to any protein of interest that contains the particularly named nucleic acid sequence and the particularly named amino acid sequence, respectively. The invention does not limit on the source (*e.g.*, cell type, tissue, animal, *etc.*), nature (*e.g.*, synthetic, recombinant, purified from cell extract, *etc.*), and/or sequence of the nucleotide sequence of interest and/or protein of interest. In one embodiment, the nucleotide sequence of interest and protein of interest include coding sequences of structural genes (*e.g.*, probe genes, reporter genes, selection marker genes, oncogenes, drug resistance genes, growth factors, *etc.*).

In one embodiment, binding of the invention's RelBκB sequences with the polypeptide sequence increases transcription of a nucleic acid sequence of interest that is operably linked to the invention's RelBκB sequences.

The terms "protein of interest," "peptide of interest," "nucleotide sequence of interest," and "molecule of interest" refer to any peptide sequence, nucleotide sequence, and molecule, respectively, the manipulation of which may be deemed desirable for any reason, by one of ordinary skill in the art. In one embodiment, the protein of interest refers to a protein encoded by a nucleic acid sequence of interest. Nucleotide sequences of interest include, but are not limited to, coding sequences of structural genes (*e.g.*, reporter genes, selection marker genes, oncogenes, drug resistance genes, growth factors, *etc.*), and non-coding regulatory sequences which do not encode an mRNA or protein product, (*e.g.*, promoter sequence, polyadenylation sequence, termination sequence, enhancer sequence, *etc.*). Illustrative genomic sequences which may be modified using the invention's methods include, but are not limited to, sequences which encode enzymes; lymphokines (*e.g.*, interleukins, interferons, TNF, *etc.*); growth factors (*e.g.*, erythropoietin, G-CSF, M-CSF,

GM-CSF, *etc.*); neurotransmitters or their precursors or enzymes responsible for synthesizing them; trophic factors (*e.g.*, BDNF, CNTF, NGF, IGF, GMF, aFGF, bFGF, NT3, NT5, HARP/pleiotrophin, *etc.*); apolipoproteins (*e.g.*, ApoAI, ApoAIV, ApoE. *etc.*); lipoprotein lipase (LPL); the tumor-suppressing genes (*e.g.*, p53, Rb, RaplA, DCC k-rev, *etc.*); factors involved in blood coagulation (*e.g.*, Factor VII, Factor VIII, Factor IX, *etc.*); suicide genes (thymidine kinase or cytosine deaminase); blood products; hormones; *etc.* In another preferred embodiment, the genomic sequences are those for which a mutant has been associated with a human disease. Such genomic sequences are exemplified, but not limited to, the adenosine deaminase (ADA) gene (GenBank Accession No. M13792) associated with adenosine deaminase deficiency with severe combined immune deficiency; alpha-1-antitrypsin gene (GenBank Accession No. M11465) associated with alpha1-antitrypsin deficiency; beta chain of hemoglobin gene (GenBank Accession No. NM_000518) associated with beta thalassemia and Sickle cell disease; receptor for low density lipoprotein gene (GenBank Accession No. D16494) associated with familial hypercholesterolemia; lysosomal glucocerebrosidase gene (GenBank Accession No. K02920) associated with Gaucher disease; hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene (GenBank Accession No. M26434, J00205, M27558, M27559, M27560, M27561, M29753, M29754, M29755, M29756, M29757) associated with Lesch-Nyhan syndrome; lysosomal arylsulfatase A (ARSA) gene (GenBank Accession No. NM_000487) associated with metachromatic leukodystrophy; ornithine transcarbamylase (OTC) gene (GenBank Accession No. NM_000531) associated with ornithine transcarbamylase deficiency; phenylalanine hydroxylase (PAH) gene (GenBank Accession No. NM_000277) associated with phenylketonuria; purine nucleoside phosphorylase (NP) gene (GenBank Accession No. NM_000270) associated with purine nucleoside phosphorylase deficiency; the dystrophin gene (GenBank Accession Nos. M18533, M17154, and M18026) associated with muscular dystrophy; the utrophin (also called the dystrophin related protein) gene (GenBank Accession No. NM_007124) whose protein product has been reported to be capable of functionally substituting for the dystrophin gene; and the human cystic fibrosis transmembrane conductance regulator (CFTR) gene (GenBank Accession No.M28668) associated with cystic fibrosis.

Exemplary molecules of interest include, but are not limited to, a peptide, glycopeptide, polysaccharide, lipopeptide, glycolipid, lipid, steroid, nucleic acid, *etc.*

The term "operably linked" when in reference to the relationship between nucleic acid sequences and/or amino acid sequences refers to linking the sequences such that they perform their intended function. For example, operably linking a promoter sequence to a nucleotide sequence of interest refers to linking the promoter sequence and the nucleotide sequence of interest in a manner such that the promoter sequence is capable of directing the transcription of the nucleotide sequence of interest and/or the synthesis of a polypeptide encoded by the nucleotide sequence of interest. The term also refers to the linkage of amino acid sequences in such a manner so that a functional protein is produced.

In another embodiment, the isolated nucleotide sequence does not bind with a protein comprising one or more of RelB, RelA, p50, RelB:p50, RelA:p50, and RelA:p52.

In one embodiment, the isolated nucleotide sequence comprises one or more of 5'-GGGAGATTTG-3' (SEQ ID NO:59) as exemplified by the sequence in the *blc-1* gene promoter (Fig 4C), 5'-GGGAGACCTG-3' (SEQ ID NO:2) as exemplified by the sequence in the *sdf-1* gene promoter, and 5'-AGGAGATTTG-3' (SEQ ID NO:60) as exemplified by the sequence in the *elc* gene promoter (Fig 4C).

The isolated nucleotide sequence that contains the RelBκB sequences of the invention include, for example, introns, exons, as well as nonsense sequences.

In one embodiment, the isolated nucleotide sequence that contains the RelBκB sequences of the invention is a probe. The term "probe" refers to an oligonucleotide (i.e., a sequence of nucleotides), whether occurring naturally as in a purified restriction digest or produced synthetically, recombinantly or by PCR amplification, that is capable of hybridizing to another oligonucleotide of interest. A probe may be single-stranded or double-stranded. Probes are useful in the detection, identification and isolation of particular gene sequences. It is contemplated that any probe used in the present invention will be labeled with any "reporter molecule," so that is detectable in any detection system, including, but not limited to fluorescent, radioactive, and luminescent systems. It is not intended that the present invention be limited to any particular detection system or label. Exemplary "probe" genes sequence (*i.e.*, sequence useful in the detection, identification and isolation of particular polypeptide sequence) encode ligand-binding systems useful for the isolation of polypeptides such as the staphylococcal protein A and its derivative ZZ (which binds to human polyclonal IgG), histidine tails (which bind to Ni^{2+}), biotin (which binds to streptavidin), maltose-binding protein (MBP) (which binds to amylose), glutathione S-transferase (which binds to glutathione), *etc.* Exemplary "reporter" gene sequences (*i.e.*

sequences that encodes a molecule such as RNA, polypeptide, *etc.*, that is detectable in enzyme-based histochemical assays, fluorescent, radioactive, and luminescent systems, *etc.*) include luciferase gene, green fluorescent protein gene, *E. coli* β-galactosidase gene, human placental alkaline phosphatase gene, and chloramphenicol acetyltransferase gene. Probes are useful in the methods disclosed below.

In another embodiment, the isolated nucleotide sequence that contains the RelBκB sequences of the invention encodes a "fusion protein," i.e., two or more polypeptides that are "operably linked" *i.e.*, wherein the linkage of nucleic acid sequences and/or amino acid sequences is such that the linked sequences perform their intended function. For example, operably linking a promoter sequence to a nucleotide sequence of interest refers to linking the promoter sequence and the nucleotide sequence of interest in a manner such that the promoter sequence is capable of directing the transcription of the nucleotide sequence of interest and/or the synthesis of a polypeptide encoded by the nucleotide sequence of interest. The term also refers to the linkage of amino acid sequences in such a manner so that a functional protein is produced. Exemplary sequences that may be linked to the invention's sequence include those for adenosine deaminase (ADA) gene (GenBank Accession No. M13792); alpha-1-antitrypsin gene (GenBank Accession No. M11465); beta chain of hemoglobin gene (GenBank Accession No. NM_000518); receptor for low density lipoprotein gene (GenBank Accession No. D16494); lysosomal glucocerebrosidase gene (GenBank Accession No. K02920); hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene (GenBank Accession No. M26434, J00205, M27558, M27559, M27560, M27561, M29753, M29754, M29755, M29756, M29757); lysosomal arylsulfatase A (ARSA) gene (GenBank Accession No. NM_000487); ornithine transcarbamylase (OTC) gene (GenBank Accession No. NM_000531); phenylalanine hydroxylase (PAH) gene (GenBank Accession No. NM_000277); purine nucleoside phosphorylase (NP) gene (GenBank Accession No. NM_000270); the dystrophin gene (GenBank Accession Nos. M18533, M17154, and M18026); the utrophin (also called the dystrophin related protein) gene (GenBank Accession No. NM_007124); and the human cystic fibrosis transmembrane conductance regulator (CFTR) gene (GenBank Accession No.M28668).

In a further embodiment, the isolated nucleotide sequence that contains the RelBκB sequences of the invention is a non-coding regulatory sequence which does not encode an mRNA or protein product, (*e.g.*, promoter sequence, polyadenylation sequence, termination sequence, enhancer sequence, *etc.*).

In a preferred embodiment, the isolated nucleotide sequence that contains the RelBκB sequences of the invention is an enhancer. The term "enhancer" refers to a *cis*-acting regulatory sequence which functions in transcription activation of genes. Activation of an enhancer results in an increase in the rate of transcription. Enhancers may be placed 5' and/or 3' to the transcription tart site, can function in either orientation, and can operate even when placed at a distance of more than 3kb from the transcriptional start site. In one embodiment, enhancers contain the consensus sequence 5'-GTGAAG-3' (SEQ ID NO:68). Exemplary enhancers include the SV40 viral enhancer. In particular, NF-κB responsive sites have been characterized in the promoters and enhancer of numerous genes (Ghosh *et al.* (1998) Ann. Rev. Immunol. 16:225-60)

In a further preferred embodiment, the isolated nucleotide sequence that contains the RelBκB sequences of the invention is a promoter. The term "promoter," "promoter element," or "promoter sequence" as used herein, refers to a DNA sequence which when ligated to a nucleotide sequence of interest is capable of controlling the transcription of the nucleotide sequence of interest into mRNA. A promoter is typically, though not necessarily, located 5' (*i.e.*, upstream) of a nucleotide sequence of interest whose transcription into mRNA it controls, and provides a site for specific binding by RNA polymerase and other transcription factors for initiation of transcription. The term "promoter" encompasses a single promoter sequence as well as to a plurality (*i.e.*, one or more) of promoter sequences which are operably linked to each other and to at least one DNA sequence of interest. For example, one of skill in the art knows that it may be desirable to use a double promoter sequence (*i.e.*, a DNA sequence containing two promoter sequences) or a triple promoter sequence (*i.e.*, a DNA sequence containing three promoter sequences) to control expression of a DNA sequence of interest. Double promoters are exemplified, but not limited to, T7-T3 (such as SEQ ID NO:69 5'-TAATACGACTCACTATAGGGATTAACCCTCACTAAAGGGA-3'), T3-T7 (such as SEQ ID NO:70 5'-ATTAACCCTCACTAAAGGGATAATACGACTCACTATAGGG-3'), T7-SP6 (such as SEQ ID NO:71 5'-TAATACGACTCACTATAGGGT ATTTAGGTGACACTATAG-3'), SP6-T7 (such as SEQ ID NO:72 5'-TATTTAGGTGACACTATAGTAATACGACTCACTATAGGG-3'), SP6-T3 (such as SEQ ID NO:73 5'-TATTTAGGTGACACTATAGATTAACCCTCACTAAAGGGA-3'), T3-SP6 (such as SEQ ID NO:74 5'-ATTAACCCTCACTAAAGGGATATTTAGGTGACACTATAG-3'), vaRNA I-tRNA,

vaRNA I-CMV, vaRNA I-RSV, vaRNA I-SV40, vaRNA I-PEPCK, vaRNA I-MT, vaRNA I-SRα, vaRNA I-P450 family, vaRNA I-GAL7, T_7-vaRNA I, T_3-vaRNA, vaRNA I-SP6, vaRNA I-K11, and vaRNA I-heat shock protein double promoters, while triple promoters are exemplified, but not limited to, the CMV-T7-vaRNA I triple promoter. The term "promoter" also covers so-called "leaky" promoters, which regulate expression of a selected DNA primarily in one tissue, but cause expression in other tissues as well.

As used herein "promoter" refers to viral, phage, prokaryotic and/or eukarytoic transcriptional control sequences. Viral promoters are exemplified by CMV, RSV, SV40, herpes simplex thymidine kinase promoter, as well as any of the various retroviral LTR promoter elements (*e.g.* the MMTV LTR).

Phage promoters are exemplified, but not limited to, promoters from T3 phage, SP6 phage, T7 phage, T5 phage, phage .phi.105, phage .phi.105MU331, lambda phage promoters (*e.g.* P_{RM} and P_R). The term "promoter" includes portions of the "full length" promoter that may be extended (*e.g.*, by PCR) to obtain a full length promoter. Thus, for example, a T7 promoter comprises 2 to 20, preferably 2 to 15, more preferably 2 to 10, yet more preferably 2 to 5 contiguous nucleotides of SEQ ID NO:75 (5'-TAATACGACTCACTATAGGG-3'). Exemplary T7 promoters may be 18 nucleotides long such as SEQ ID NO:76 (5'-TAATACGACTCACTATA-3') (see U.S. Patent Application No. 20020068290A1, to Yarovinsky, Timur, June 6, 2002), 16 nucleotides long such as SEQ ID NO:77 (5'-TAATACGACTCACTAT-3'), SEQ ID NO:78 (5'-AATACGACTCACTATA-3'), and SEQ ID NO:79 (5'-ATACGACTCACTATAG-3'), 14 nucleotides long such as SEQ ID NO:80 (5'-ACGACTCACTATAG-3'), SEQ ID NO:81 (5'-ATACGACTCACTAT-3'), and SEQ ID NO:82 (5'-TACGACTCACTATA-3'), and 10 nucleotides long such as SEQ ID NO:83 (5'-CGACTCACTA-3'), SEQ ID NO:84 (5'-TACGACTCAC-3'), and SEQ ID NO:85 (5'-ATACGACTCA-3'). In a preferred embodiment, the T7 promoter is a hexanucleotide exemplified by SEQ ID NO:86 (5'-ATAGGG-3'), (Figure 2), SEQ ID NO:87 (5'-TAATAC-3'), and SEQ ID NO:88 (5'-ACGACT-3').

In yet another example, a T3 promoter comprises 2 to 20, preferably 2 to 15, more preferably 2 to 10, yet more preferably 2 to 5 contiguous nucleotides of SEQ ID NO:89 (5'-ATTAACCCTCACTAAAGGGa-3'). Exemplary T3 promoters may be 18 nucleotides long such as SEQ ID NO:90 (5'-TTAACCCTCACTAAAGGG-3'), SEQ ID NO:91 (5'-TAACCCTCACTAAAGGGA-3'), and SEQ ID NO:92

(5'-ATTAACCCTCACTAAAGG-3'), 16 nucleotides long such as SEQ ID NO:93 (5'-ACCCTCACTAAAGGGA-3'), SEQ ID NO:94 (5'-TAACCCTCACTAAAGG-3'), and SEQ ID NO:95 (5'-TTAACCCTCACTAAAG-3'), 14 nucleotides long such as SEQ ID NO:96 (5'-CCTCACTAAAGGGA-3'), SEQ ID NO:97 (5'-ATTAACCCTCACTA-3'), and SEQ ID NO:98 (5'-AACCCTCACTAAAG-3'), 10 nucleotides long such as SEQ ID NO:99 (5'-ACCCTCACTA-3'), SEQ ID NO:100 (5'-CCTCACTAAA-3'), and SEQ ID NO:101 (5'-ATTAACCCTC-3'), and 6 nucleotides long such as SEQ ID NO:102 (5'-ATTAAC-3'), SEQ ID NO:103 (5'-AAGGGA-3'), and SEQ ID NO:104 (5'-CACTAA-3').

In a further example, a SP6 promoter comprises 2 to 19, preferably 2 to 15, more preferably 2 to 10, yet more preferably 2 to 5 contiguous nucleotides of SEQ ID NO:105 (5'-TATTTAGGTGACACTATAG-3'). Exemplary SP6 promoters may be 16 nucleotides long such as SEQ ID NO:106 (5'-TTAGGTGACACTATAG-3'), SEQ ID NO:107 (5'-TTTAGGTGACACTATA-3'), and SEQ ID NO:108 (5'-TATTTAGGTGACACTA-3'), 14 nucleotides long such as SEQ ID NO:109 (5'-ATTTAGGTGACACT-3'), SEQ ID NO:110 (5'-TATTTAGGTGACAC-3'), and SEQ ID NO:111 (5'-TTTAGGTGACACTA-3'), 10 nucleotides long such as SEQ ID NO:112 (5'-TTAGGTGACA-3'), SEQ ID NO:113 (5'-TAGGTGACAC-3'), and SEQ ID NO:114 (5'-ATTTAGGTGA-3'), and 6 nucleotides long such as SEQ ID NO:115 (5'-CTATAG-3'), SEQ ID NO:116 (5'-TATTTA-3'), and SEQ ID NO:117 (5'-GACACT-3').

Prokaryotic promoters include those carrying optimal -35 and -10 (Pribnow box) sequences for transcription by a prokaryotic (*e.g. E. coli*) RNA polymerase. In addition, some prokaryotic promoters contain overlapping binding sites for regulatory repressors (*e.g.* the Lac promoter and the synthetic TAC promoter, which contain overlapping binding sites for lac repressor thereby conferring inducibility by the substrate homolog IPTG). Prokaryotic genes from which suitable promoters sequences may be obtained include the *E. coli* lac, ara and trp genes. Further exemplary promoters include, PEPCK, MT, SRα, P450 family, GAL7, K11, and heat shock protein promoters.

In particularly preferred embodiments, the promoter is chosen from one or more of *Sdf*-1 promoter, *Blc* promoter, *Elc* promoter, and *Slc* promoter.

It is expressly contemplated that the invention RelBκB sequences may be in any orientation. Thus, reference to 5'-NGGAGANNTG-3' (SEQ ID NO:57) includes within its scope sequences in the reverse orientation, *i.e.*, 5'-GTNNAGAGGN-3' (SEQ ID NO:118).

It is also contemplated that where the invention's RelBκB sequences are contained within a nucleotide sequence such as probe, enhancer, promoter, intron, exon, and nonsense sequence, the invention's RelBκB sequences may be in the middle, closer to the 5' end than to the 3' end, and/or closer to the 3' end than to the 5' end, of the nucleotide sequence. For example, data herein shows that the invention's RelBκB sequence is closer to the 5' end than the 3' end of the *blc-1* gene promoter (Figure 4C), and is close to the 3' end than the 5' end of the *sdf-1* gene promoter.

It is further contemplated that a nucleotide sequence contain one or more of the invention's RelBκB sequences, preferably from 1 to 20, more preferably from 1 to 15, yet more preferably from 1 to 10, and most preferably from 1 to 5, of the invention's RelBκB sequences. Further, it is contemplated that, where more than one of the inventions" RelBκB sequences is present, these sequences may be the same or different.

In one embodiment, the invention's RelBκB sequences specifically bind with a polypeptide sequence comprising binds with a polypeptide sequence comprising a RelB Rel homology domain (RHD). In one preferred embodiment, the RelB RHD is exemplified by SEQ ID NO:62, *i.e.*, amino acids 1-400 of the exemplary mouse RelB shown in Figure 13, GenBank accession A42023. In a more preferred embodiment, SEQ ID NO:62 is contained in one or more of RelB and RelB:p52.

The terms "specific binding," "binding specificity," and grammatical equivalents thereof when made in reference to the binding of a first molecule (such as a polypeptide, glycoprotein, nucleic acid sequence, *etc.*) to a second molecule (such as a polypeptide, glycoprotein, nucleic acid sequence, *etc.*) refer to the preferential interaction between the first molecule with the second molecule as compared to the interaction between the second molecule with a third molecule. Specific binding is a relative term that does not require absolute specificity of binding; in other words, the term "specific binding" does not require that the second molecule interact with the first molecule in the absence of an interaction between the second molecule and the third molecule. Rather, it is sufficient that the level of interaction between the first molecule and the second molecule is higher than the level of interaction between the second molecule with the third molecule. "Specific binding" of a first molecule with a second molecule also means that the interaction between the first molecule and the second molecule is dependent upon the presence of a particular structure on or within the first molecule; in other words the second molecule is recognizing and binding to a specific structure on or within the first molecule rather than to nucleic acids or

to molecules in general. For example, if a second molecule is specific for structure "A" that is on or within a first molecule, the presence of a third nucleic acid sequence containing structure A will reduce the amount of the second molecule which is bound to the first molecule. The conditions for binding molecules may be determined using routine methods, commercially available methods, and methods disclosed herein.

E. Methods for identifying test compounds that alter RelB DNA-binding activity

The invention further provides methods for identifying one or more test compounds that alter binding of RelB Rel homology domain (RelB RHD) with a RelBκB sequence of the present invention. In one embodiment, the invention's methods comprise: a) contacting i) the isolated nucleotide sequence comprising RelBκB sequences described *supra* with ii) a polypeptide comprising RelB RHD, as exemplified by SEQ ID NO:62, in the presence and absence of the one or more test compounds; and detecting altered specific binding of the nucleotide sequence with RelB RHD, as exemplified by SEQ ID NO:62, in the presence of the one or more test compounds compared to in the absence of the one or more test compounds, and c) identifying the one or more test compounds as altering binding of RelB RHD with a RelBκB sequence. In one embodiment, it may be desirable to use a control such as non-activatable form of IKKα.

The terms "RelB DNA-binding activity" and "IKKα mediated cellular activity" are used interchangeably to refer to the binding of RelB Rel homology domain (RelB RHD) with the invention's RelBκB sequence.

The terms "NF-κB2," "NF-κ B2," "NFκB2," "NF-κB2/p100," "NF-κ B2/p100," "NFκB2/p100," "p100," "p52/p100," "p49/p100," "lyt," "lyt10, "lyt-10," are used herein interchangeably to refer to a polypeptide that is a precursor of a transcription factor, and that contains an N-terminal Rel homology domain (RHD) that is common to all NF-κB proteins as well as an inhibitory IκB-like C-terminal domain of ankyrin repeats (Ghosh *et al.* (1998) Ann Rev Immunol 16, 225-260). Exemplary NF-κB2 sequences include those from human (Figures 8, 9), mouse (Figures 10, 11), Rhesus monkey (see, Figure 12)

The terms "NF-κB2/p52," "NF-κ B2/p52," "p49," "p52," "p50B," are used herein interchangeably to refer to a transcription factor that contains a DNA-binding protein, and that is the cleavage product resulting from the ubiquitin-dependent proteolytic digestion of

NF-κ B2, which remove the C-terminal domain of NF-κ B2, leaving the RHD. This degradation is caused by the protein kinase NIK and is mediated by phosphorylation of NF-κ B2 by IKKα.

The test compounds identified by the invention's methods refer to any type of molecule (for example, a peptide, nucleic acid, carbohydrate, lipid, organic, and inorganic molecule, *etc.*) obtained from any source (for example, plant, animal, and environmental source, *etc.*), or prepared by any method (for example, purification of naturally occurring molecules, chemical synthesis, and genetic engineering methods, *etc.*). The terms "agent," "test agent," "molecule," "test molecule," "compound," and "test compound" as used interchangeably herein. Test compounds are exemplified by, but not limited to, antibodies, nucleic acid sequences, and other agents as further described below.

In one preferred embodiment, the agent that alters binding of RelB RHD with a RelBκB sequence is an antibody, such as RelB RHD antibody, and/or RelBκB sequence antibody. The terms "antibody" and "immunoglobulin" are interchangeably used to refer to a glycoprotein or a portion thereof (including single chain antibodies), which is evoked in an animal by an immunogen and which demonstrates specificity to the immunogen, or, more specifically, to one or more epitopes contained in the immunogen. The term "antibody" includes polyclonal antibodies, monoclonal antibodies, naturally occurring antibodies as well as non-naturally occurring antibodies, including, for example, single chain antibodies, chimeric, bifunctional and humanized antibodies, as well as antigen-binding fragments thereof, including, for example, Fab, $F(ab')_2$, Fab fragments, Fd fragments, and Ev fragments of an antibody, as well as a Fab expression library. It is intended that the term "antibody" encompass any immunoglobulin (*e.g.*, IgG, IgM, IgA, IgE, IgD, *etc.*) obtained from any source (*e.g.*, humans, rodents, non-human primates, caprines, bovines, equines, ovines, *etc.*). The term "polyclonal antibody" refers to an immunoglobulin produced from more than a single clone of plasma cells; in contrast "monoclonal antibody" refers to an immunoglobulin produced from a single clone of plasma cells. Monoclonal and polyclonal antibodies may or may not be purified. For example, polyclonal antibodies contained in crude antiserum may be used in this unpurified state.

Naturally occurring antibodies may be generated in any species including murine, rat, rabbit, hamster, human, and simian species using methods known in the art. Non-naturally occurring antibodies can be constructed using solid phase peptide synthesis,

can be produced recombinantly or can be obtained, for example, by screening combinatorial libraries consisting of variable heavy chains and variable light chains as previously described [Huse *et al.*, Science 246:1275-1281 (1989)]. These and other methods of making, for example, chimeric, humanized, CDR-grafted, single chain, and bifunctional antibodies are well known to those skilled in the art (Winter and Harris, Immunol. Today 14:243-246 (1993); Ward *et al.*, Nature 341:544-546 (1989); Hilyard *et al.*, Protein Engineering: A practical approach (IRL Press 1992); and Borrabeck, Antibody Engineering, 2d ed. (Oxford University Press 1995).

Those skilled in the art know how to make polyclonal and monoclonal antibodies which are specific to a desirable polypeptide. For the production of monoclonal and polyclonal antibodies, various host animals can be immunized by injection with the peptide corresponding to any molecule of interest in the present invention, including but not limited to rabbits, mice, rats, sheep, goats, *etc.* In one preferred embodiment, the peptide is conjugated to an immunogenic carrier (*e.g.*, diphtheria toxoid, bovine serum albumin (BSA), or keyhole limpet hemocyanin (KLH)). Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (Bacille Calmette-Guerin) and *Corynebacterium parvum.*

For preparation of monoclonal antibodies directed toward molecules of interest in the present invention, any technique that provides for the production of antibody molecules by continuous cell lines in culture may be used (*See, e.g.*, Harlow and Lane, *Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press*, Cold Spring Harbor, NY). These include but are not limited to the hybridoma technique originally developed by Köhler and Milstein (Köhler and Milstein, Nature 256:495-497 [1975]), as well as the trioma technique, the human B-cell hybridoma technique (*See e.g.*, Kozbor *et al.* Immunol. Today 4:72 [1983]), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole *et al., in Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96 [1985]). In some particularly preferred embodiments of the present invention, the present invention provides monoclonal antibodies of the IgG class.

In additional embodiments of the invention, monoclonal antibodies can be produced in germ-free animals utilizing technology such as that described in PCT/US90/02545. In

addition, human antibodies may be used and can be obtained by using human hybridomas (Cote *et al.*, Proc. Natl. Acad. Sci. U.S.A.80:2026-2030 [1983]) or by transforming human B cells with EBV virus in vitro (Cole *et al.*, *in Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, pp. 77-96 [1985]).

Furthermore, techniques described for the production of single chain antibodies (*See e.g.*, U.S. Patent 4,946,778; herein incorporated by reference) can be adapted to produce single chain antibodies that specifically recognize a molecule of interest (*e.g.*, at least a portion of an AUBP or mammalian exosome, as described herein). An additional embodiment of the invention utilizes the techniques described for the construction of Fab expression libraries (Huse *et al.*, Science 246:1275-1281 [1989]) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity for a particular protein or epitope of interest (*e.g.*, at least a portion of an AUBP or mammalian exosome).

The invention also contemplates humanized antibodies. Humanized antibodies may be generated using methods known in the art, including those described in U.S. Patent Numbers 5,545,806; 5,569,825 and 5,625,126, the entire contents of which are incorporated by reference. Such methods include, for example, generation of transgenic non-human animals which contain human immunoglobulin chain genes and which are capable of expressing these genes to produce a repertoire of antibodies of various isotypes encoded by the human immunoglobulin genes.

According to the invention, techniques described for the production of single chain antibodies (U.S. 4,946,778; herein incorporated by reference) can be adapted to produce specific single chain antibodies as desired. An additional embodiment of the invention utilizes the techniques known in the art for the construction of Fab expression libraries (Huse *et al.*, Science, 246:1275-1281 [1989]) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

Antibody fragments that contain the idiotype (antigen binding region) of the antibody molecule can be generated by known techniques. For example, such fragments include but are not limited to: the F(ab')2 fragment that can be produced by pepsin digestion of an antibody molecule; the Fab' fragments that can be generated by reducing the disulfide bridges of an F(ab')2 fragment, and the Fab fragments that can be generated by treating an antibody molecule with papain and a reducing agent.

In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art (*e.g.*, radioimmunoassay, ELISA

[enzyme-linked immunosorbent assay], "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitin reactions, immunodiffusion assays, *in situ* immunoassays [*e.g.*, using colloidal gold, enzyme or radioisotope labels], Western blots, precipitation reactions, agglutination assays (*e.g.*, gel agglutination assays, hemagglutination assays, *etc.*), complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, *etc.*

In an alternative embodiment, the agent that alters the level of binding of RelB RHD with a RelBκB sequence is a nucleic acid sequence. The terms "nucleic acid sequence" and "nucleotide sequence" as used herein refer to two or more nucleotides which are covalently linked to each other. Included within this definition are oligonucleotides, polynucleotide, and fragments or portions thereof, DNA or RNA of genomic or synthetic origin which may be single- or double-stranded, and represent the sense or antisense strand. Nucleic acid sequences which are particularly useful in the instant invention include, without limitation, antisense sequences and ribozymes.

In one embodiment, the agent that alters the level of binding of RelB RHD with a RelBκB sequence is an antisense nucleic acid sequence. Antisense sequences have been successfully used to inhibit the expression of several genes [Markus-Sekura (1988) Anal. Biochem. 172:289-295; Hambor *et al.* (1988) J. Exp. Med. 168:1237-1245; and patent EP 140 308], including the gene encoding VCAM1, one of the integrin α4β1 ligands [U.S. Patent No. 6,252,043, incorporated in its entirety by reference]. The terms "antisense DNA sequence" and "antisense sequence" as used herein interchangeably refer to a deoxyribonucleotide sequence whose sequence of deoxyribonucleotide residues is in reverse 5' to 3' orientation in relation to the sequence of deoxyribonucleotide residues in a sense strand of a DNA duplex. A "sense strand" of a DNA duplex refers to a strand in a DNA duplex which is transcribed by a cell in its natural state into a "sense mRNA." Sense mRNA generally is ultimately translated into a polypeptide. Thus, an "antisense DNA sequence" is a sequence which has the same sequence as the non-coding strand in a DNA duplex, and which encodes an "antisense RNA" (*i.e.*, a ribonucleotide sequence whose sequence is complementary to a "sense mRNA" sequence). The designation (-) (*i.e.*, "negative") is sometimes used in reference to the antisense strand, with the designation (+) sometimes used in reference to the sense (*i.e.*, "positive") strand. Antisense RNA may be produced by any method, including synthesis by splicing an antisense DNA sequence to a promoter which permits the synthesis of antisense RNA. The transcribed antisense RNA

strand combines with natural mRNA produced by the cell to form duplexes. These duplexes then block either the further transcription of the mRNA or its translation, or promote its degradation.

Antisense oligonucleotide sequences may be synthesized using any of a number of methods known in the art (such as solid support and commercially available DNA synthesizers, standard phosphoramidate chemistry techniques, and commercially available services, *e.g.*, Genta, Inc.).

In some alternative embodiments, the agent that alters the level of binding of RelB RHD with a RelBκB sequence is a ribozyme nucleic acid sequence. Ribozyme sequences have been successfully used to inhibit the expression of several genes including the gene encoding VCAM1, which is one of the integrin α4β1 ligands [U.S. Patent No. 6,252,043, incorporated in its entirety by reference]. The term "ribozyme" refers to an RNA sequence that hybridizes to a complementary sequence in a substrate RNA and cleaves the substrate RNA in a sequence specific manner at a substrate cleavage site. Typically, a ribozyme contains a "catalytic region" flanked by two "binding regions." The ribozyme binding regions hybridize to the substrate RNA, while the catalytic region cleaves the substrate RNA at a "substrate cleavage site" to yield a "cleaved RNA product."

Molecules which find use as agents for specifically altering the level of specific binding of RelB RHD with a RelBκB sequence include organic molecules, inorganic molecules, and libraries of any type of molecule, which can be screened using a method of the invention, and which may be prepared using methods known in the art. These agents are made by methods for preparing oligonucleotide libraries [Gold *et al.*, U.S. Patent No. 5,270,163, incorporated by reference]; peptide libraries [Koivunen *et al.* J. Cell Biol., 124: 373-380 (1994)]; peptidomimetic libraries [Blondelle *et al.*, Trends Anal. Chem. 14:83-92 (1995)] oligosaccharide libraries [York *et al.*, Carb. Res. 285:99-128 (1996); Liang *et al.*, Science 274:1520-1522 (1996); and Ding *et al.*, Adv. Expt. Med. Biol. 376:261-269 (1995)]; lipoprotein libraries [de Kruif *et al.*, FEBS Lett., 399:232-236 (1996)]; glycoprotein or glycolipid libraries [Karaoglu *et al.*, J. Cell Biol. 130:567-577 (1995)]; or chemical libraries containing, for example, drugs or other pharmaceutical agents [Gordon *et al.*, J. Med. Chem. 37:1385-1401 (1994); Ecker and Crook, Bio/Technology 13:351-360 (1995), U.S. Patent No. 5,760,029, incorporated by reference]. Libraries of diverse molecules also can be obtained from commercial sources.

The invention's methods identify one or more test compounds that alter binding of RelB Rel homology domain (RelB RHD) to the invention's nucleotides sequences. The term "Rel" refers to a family of reticuloendotheliosis proteins (such as RelA, RelB, p50, p52, v-rel, c-rel, *etc.*). "RelB" is exemplified by mouse sequences shown in Figures 13-15, and Xenopus Laevis sequences shown in Figures 16-17. "RelA" is also known as "p65" and is exemplified by sequences shown in Figures 20 and 21. Exemplary "NFκ-B1" also referred to as "p105" that are processed into "p50" are isolated from mouse (Figures 18, and 19).

"Rel homology domain," "RHD," "DNA-binding domain," and "DBD," are used interchangeably herein to refer to a polypeptide sequence on a member of the Rel family of proteins (such as RelA, RelB, p50, p52, v-rel, c-rel, *etc.)* that is at the N-terminal end of the polypeptide sequence of the Rel family protein member, that binds to a target DNA sequence (such as the consensus sequence 5'-GGGGGYNNCCY-3' (SEQ ID NO:119) and/or to one or more of the invention's RelBκB sequences), that is involved in binding to a member of the Rel family of proteins in forming homodimers (such as p52:p52, p50-p50, *etc.*) and/or heterodimers (such as RelB-p52, RelA:p50, *etc.*), and/or that is involved in binding to one or more IκB family members (such as IκB, IκBα, IκBβ, IκBε, IκBγ, and Bcl-3; Ghosh *et al.* (1998) Annu. Rev. Immunol. 16:225-60).

In one embodiment, the RHD is about 400 amino acids. In another embodiment, the RHD is about 350 amino acids. In yet another embodiment, the RHD is about 300 amino acids. Examples of the RHD include the RHD of human p52 (SEQ ID NO:64, *i.e.*, residues 1-340 of GenBank accession NM 002502 in Figure 8, RHD of murine RelB (SEQ ID NO:62, *i.e.*, residues 1-400 of GenBank accession A42023 in Figure 13, and RelB RHD disclosed in Ghosh *et al.* (1998) Ann. Rev. Immunol. 16:225-60.

The RHD contains the "dimerization domain," also known as "DD". The dimerization domain is exemplified by the RelB DD listed as SEQ ID NO:63.

In one embodiment, the invention's methods may further comprise d) contacting the nucleotide sequence, in the presence of the one or more test compounds, with one or more compositions comprising a polypeptide that comprises RelB RHD, as exemplified by SEQ ID NO:62. The term "control" as used herein when in reference to a sample, cell, tissue, animal, *etc.*, refers to any type of sample, cell, tissue, animal, *etc.* that one of ordinary skill in the art may use for checking the results of another sample, cell, tissue, animal, *etc.*, by

maintaining the same conditions except in some one particular factor, and thus inferring the causal significance of this varied factor.

In one embodiment, In one embodiment, it may be desirable to use a control such as non-activatable form of IKKα. In such an embodiment, the invention's methods may further comprise d) contacting the nucleotide sequence, in the presence of the one or more test compounds, with one or more compositions comprising a polypeptide that comprises RelB RHD, as exemplified by SEQ ID NO:62, wherein the composition comprising a polypeptide that comprises RelB RHD, as exemplified by SEQ ID NO:62, is chosen from one or more of cell extract, cytoplasmic extract, and nuclear extract, and wherein the composition is isolated from a mammalian cell comprising IKKα having reduced kinase activity, and the mammalian cell is treated with one or more IKKα activators chosen from lymphotoxin B (LTβ), B cell activating factor belonging to the TNF family (BAFF), anti-LTβR antibody, and CD40 ligand (CD40L); and e) detecting unaltered specific binding of the isolated nucleotide sequence with RelB RHD, as exemplified by SEQ ID NO:62, in the presence and absence of the one or more test compounds.

The term "mammal" refers to the exemplary animals of rodent, primate (including simian and human) ovine, bovine, ruminant, lagomorph, porcine, caprine, equine, canine, feline, ave, *etc.* Preferred mammals are selected from the order Rodentia, such as mouse and rat.

The term "IKKα" refers to the exemplary human and mouse amino acids shown in Figures 22 and 24, respectively, which are encoded by the exemplary nucleic acid sequences of Figures 23 and 25, respectively. IKKα contains a "IKKα kinase domain," which is also referred to as "IKKα protein kinase domain," and "IKKα protein kinase catalytic domain," and which refers to amino acids 1 to 301 of the human IKKα kinase (Figure 22), and/or to amino acids 1 to 301 of the mouse IKKα kinase (Figure 24), and/or portions thereof that exhibit IKKα kinase activity.

Various methods are known in the art for generating transgenic animals that express reduced IKKα activity. For example, transgenic animals expressing non-activatable IKKα. Exemplary animals include knockout mice in which at least a portion of the *ikkα* gene is knocked out (Hu *et al.* (1999) Science 284:316-320; Takeda *et al.* (1999) Science 284:313-316); knonckin mice such as $Ikk\alpha^{AA/AA}$ mice, which express a non-activatable form of IKKα as a result of the introduction of a knockin $Ikk\alpha^{AA}$ allele that inactivates the

kinase activity of IKKα (Cao *et al.* (2001) Cell 107, 763-775); transgenic mice expressing an IKKα kinase domain in which Serine at one or more of amino acids 176 and 180 the of IKKα kinase polypeptide is replaced with Alanine; and/or in which Lysine at amino acid 44 of IKKα kinase polypeptide is replaced with Alanine or Methionine (Delhase *et al.* (1999) Science 284:309); other mutations are also known in the art to inactivate IKKα.

In one embodiment, the polypeptide comprising RelB RHD is recombinant. In a preferred embodiment, the polypeptide comprising the exemplary SEQ ID NO:62 is RelB and/or RelB:p52. In a yet more preferred embodiment, the polypeptide comprising the exemplary SEQ ID NO:62 is chosen from one or more of RelB and RelB:p52, and is isolated from a mammalian cell treated with one or more IKKα activators chosen from lymphotoxin B (LTβ), B cell activating factor belonging to the TNF family (BAFF), anti-LTβR antibody, and CD40 ligand (CD40L).

The term "IKKα activator" refers to a compound that allows phosphorylation of IKKα, and expression of IKKα kinase activity. In one embodiment, such a compound also allows expression of IKKβ kinase activity. However, in a preferred embodiment, the IKKα activator results in a greater statistically significant increase in IKKα kinase activity compared to an increase in IKKβ kinase activity. Exemplary IKKα activators include, without limitation, lymphotoxin (LT) α_1:β_2 trimers acting through LTβ receptor (LTβR), B cell activating factor (BAFF; also known as Blys/TALL-1/THANK and B cell activating factor belonging to the TNF family) acting through BAFF-R and CD40 ligand (CD40L) whose receptor is CD40 (Claudio *et al.* (2002) Nat Immunol 3, 958-965; Coope *et al.* (2002) EMBO J 21, 5375-5385; Dejardin *et al.* (2002) Immunity 17, 525-535; Kayagaki *et al.* (2002) Immunity 17, 515-524; Yilmaz *et al.* (2003) EMBO J 22, 121-130).

In one embodiment, the mammalian cell is *in vivo* (for example, in an animal that harbors a recombinant expression vector or that contains wild type homologous sequences). Alternatively, the mammalian cell is *in vitro* (for example, cells that harbors a recombinant expression vector or that contains wild type homologous sequences). The mammalian cell may be a primary cell or from a cell line. Furthermore, the mammalian cell may be chosen from one or more of B cell, stromal cell of lymph organ such as spleen, fibroblast cell such as embryo fibroblasts (EFs), including mouse embryo fibroblasts (MEFs), macrophage cell such as stromal macrophage cell, dendritic cell, neuron cell, plasma cell, lymphoid cell, lymphoblastoid cell, myeloid cell, Reed-Sternber (HRS) cell of Hodgkin's lymphomas,

epithelial cell such as breast cell, gastric cell, lung cell, prostate cell, cervical cell, pancreatic cell, colon cell, rectal cell, ovarian cell, stomach cell, esophagus cell, mouth cell, tongue cell, gum cell, skin cell, muscle cell, heart cell, liver cell, bronchial cell, cartilage cell, bone cell, testis cell, kidney cell, endometrium cell, uterus cell, bladder cell, thyroid cell, brain cell, gall bladder cell, and ocular cell (such as cell of the cornea, cell of uvea, cell of the choroids, cell of the macula, vitreous humor cell, etc.).

In another alternative embodiment, it may be desirable to use RelA:p52 as control. Thus, the method further comprises detecting unaltered binding of the isolated nucleotide sequence to one or more proteins chosen from a polypeptide comprising one or more of RelA RHD, RelA, p52, and RelA:p52, in the presence and absence of the one or more test compounds.

In a further alternative, it may be desirable to use RelB:p50 as a control. Thus, the method further comprises detecting unaltered binding of the isolated nucleotide sequence to one or more proteins chosen from a polypeptide comprising one or more of RelB RHD, RelB, p50, and RelB:p50, in the presence and absence of the one or more test compounds.

Alternatively, the consensus-κB sequence may be used as a control. Thus, the method further comprises detecting unaltered binding of an isolated nucleotide sequence comprising the consensus-κB sequence 5'-GGGACTTTCC-3' (SEQ ID NO:58) to a polypeptide comprising one or more of RelB RHD, as exemplified by SEQ ID NO:62, and RelB in the presence of the one or more test compounds.

The methods may further comprise identifying the one or more test compounds as altering symptoms associated with IKKα related pathology.

The invention's methods involve detecting the level of specific binding of the isolated nucleotide sequence with RelB RHD, as exemplified by SEQ ID NO:62. Methods for "detecting" such binding are known in the art and disclosed herein. For example, binding of polypeptides (including dimerized polypeptides such as RelB:p52 and RelA:p50) to DNA molecules may be determined by methods including, but are not limited to, direct binding in solution, direct binding where one or more components is immobilized on a solid surface, electrophoretic mobility shift assays, nucleolytic cleavage protection assays such as DNAse I footprinting assay, reporter gene assay, optical affinity biosensor system assay, PCR-based target detection assay, chemical footprinting assay, filter binding assay, immunological assay, sedimentation centrifugation assay, spectroscopic assay, HPLC and other column and thin layer chromatographic assays, immunologic detection assays such as

ELISA, tagged antibody, and precipitation assays. These exemplary methods are disclosed in the art such as in U.S. Patent Nos. 5,783,384, 6,333,153.

In a preferred embodiment, detecting the level of specific binding of the isolated nucleotide sequence that contains the invention's RelBκB sequences (*e.g.*, SEQ ID NO:57) with the protein RelB RHD, as exemplified by SEQ ID NO:62, employs arrays, electrophoretic mobility shift assay (EMSA), immunoprecipitation, ELISA, footprinting assay, reporter gene assay, optical affinity biosensor system assays and the like. One of skill in the art that these exemplary methods are also useful for detecting specific binding of the isolated nucleotide sequence that contains the invention's RelBκB sequences (*e.g.*, SEQ ID NO:57) with the protein RelB RHD, as exemplified by SEQ ID NO:62, are also useful in screening test compounds that alter such binding.

F. Detection of specific binding, and screening test compounds, using arrays

The level of specific binding of the isolated nucleotide sequence that contains the invention's RelBκB sequences (*e.g.*, SEQ ID NO:57) with the protein RelB RHD, as exemplified by SEQ ID NO:62, may be determined using an "array", *i.e.*, a plurality (*i.e.*, more than one) of reaction compartments. In one embodiment, each of the reaction compartments comprises one test compound. More preferably, the test compound in each of the reaction compartments is different from the test compound in other reaction compartments. Alternatively, some of the reaction compartments may contain the same test compound, *e.g.*, for duplicate/triplicate testing of the same test compound. In one embodiment, the plurality of reaction compartments comprises a micro-well titre plate, also called microplate, such as those used in binding assays, *e.g.*, an ELISA assay, receptor binding and nucleic acid probe hybridization techniques. Binding may be determined by mixing the components of the reaction mixture in solution, or binding of one or more components that are in solution to one or more surface bound (i.e., immobilized) molecules. In one embodiment, the plurality of reaction compartments comprises at least 48 or at least 96 of the reaction compartments.

Apparatus and methods for using arrays to facilitate screening of a large number of test compounds are known in the art. For example, Santini Jr. *et al.* U.S. Patent No. 6,551,838 discloses microfabricated devices for the storage and selective exposure of chemicals and devices. U.S. Pat. No. 5,843,767 to Beattie discloses a microfabricated, flowthrough "genosensors" for the discrete detection of binding reactions. The apparatus

includes a nanoporous glass wafer having tapered wells in which nucleic acid recognition elements are immobilized. U.S. Pat. No. 6,083,763 to Balch discloses an apparatus for analyzing molecular structures within a sample substance using an array having a plurality of test sites upon which the sample substance is applied. The test sites typically are in microplate arrays, such as microtitre plates. U.S. Pat. No. 5,797,898 and U.S. Pat. No. 6,123,861 to Santini, *et al.* describe microchip devices that release drug molecules from reservoirs having reservoir caps that actively or passively disintegrate. U.S. Pat. No. 5,252,294 to Kroy discloses micromechanical structures having closed cavities for use in storage and handling of substances, for example, in research and testing of the substances.

G. Detection of specific binding, and screening test compounds, using Electrophoretic mobility shift (EMS) assays

The level of specific binding of the isolated nucleotide sequence that contains the invention's RelBκB sequences (*e.g.*, SEQ ID NO:57) with the protein RelB RHD, as exemplified by SEQ ID NO:62, may be determined using electrophoretic mobility shift assays, also known as gel retardation assays. In gel retardation assays, a double stranded synthetic oligonucleotide is constructed, having the specific nucleotide sequence to that the sequence-specific DNA-binding protein binds. The oligonucleotide is labeled, usually with radioactive phosphate, and incubated with the preparation containing the DNA-binding protein. The oligonucleotide is then electrophoresed on a suitable gel. The binding of a sequence-specific DNA-binding protein is detected as a retardation in the migration of the radioactive oligonucleotide.

Exemplary EMS assays are disclosed herein (Figure 4B, 5A, 5B) and known in art such as in U.S. Patent Nos. 6,333,153, 5,900,358, 6,548,540, and 6,150,090, incorporated by reference. Briefly, a nucleic acid binding protein is contacted with a target nucleic acid sequence under suitable conditions to promote specific binding between the protein and the target nucleic acid sequence, electrophoresing the mixture, and detecting the amount of and/or location of the protein and/or the labeled target nucleic acid sequence. The protein may be detected using antibodies, while the target nucleic acid sequence may be detected by detecting a label attached thereto. The "label" may be radioactive or non-radioactive (such as a fluorescent molecule, a chemiluminescent molecule, and biotin). Methods for non-radioactive gel shift assays are known in the art such as those in U.S. patent no. 5,900,358.

H. Detection of specific binding, and screening test compounds, using footprinting Assays

The level of specific binding of the isolated nucleotide sequence that contains the invention's RelBκB sequences (*e.g.*, SEQ ID NO:57) with the protein RelB RHD, as exemplified by SEQ ID NO:62, may be determined using footprinting assays. Generally, "footprinting" assays involve binding of a DNA binding protein to a target nucleic acid sequence, which protects the bound portion of the nucleic acid sequence from subsequent nuclease digestion (*e.g.*, using DNase I). More particularly, in foot printing assays, a radioactively-labeled DNA molecule containing the DNA sequence (such as SEQ ID NO:57) to which the sequence-specific DNA-binding molecule (*e.g.*, RelB RHD as exemplified by SEQ ID NO:62) binds is incubated with the sequence-specific DNA-binding molecule and then digested with DNAaseI, an enzyme that cuts DNA molecules regardless of their sequence. This forms DNA fragments of all possible lengths that can be separated by sequencing gel electrophoresis. A "ladder" of these different length fragments is formed on the gel. Binding of the sequence-specific DNA-binding molecule to its cognate nucleotide recognition sequence protects the DNA in that region from digestion with DNAaseI. This protection is observed as a region of reduced intensity of radioactivity on a sequencing gel, *i.e.*, the "ladder" is missing contiguous "rungs" where the protein was bound.

Methods for footprinting assays are known in the art such as those disclosed in U.S. Patent Nos. 5,510,256, 6,548,734, 6,537,810, and 6,150,090.

I. Detection of specific binding, and screening test compounds, using reporter gene assays

The level of specific binding of the isolated nucleotide sequence that contains the invention's RelBκB sequences (*e.g.*, SEQ ID NO:57) with the protein RelB RHD, as exemplified by SEQ ID NO:62, may be determined using reporter gene assays. Methods for reporter gene assays are known in art such as those disclosed in U.S. Patent Nos. 6,537,973 and 6,150,090, incorporated by reference.

In one embodiment, the invention provides a method for identifying one or more test compounds that alters binding of RelB Rel homology domain (RelB RHD) with a RelBκB sequence, comprising: a) contacting i) an isolated nucleotide sequence containing the

invention's RelBκB sequences (*e.g.*, SEQ ID NO:57), that is operably linked to a nucleic acid sequence encoding a reporter molecule with ii) a polypeptide comprising RelB Rel homology domain (RelB RHD, as exemplified by SEQ ID NO:62), such that RelB RHD specifically binds with the invention's RelBκB sequences (*e.g.*, SEQ ID NO:57), wherein the contacting is in the presence and absence of the one or more test compounds; b) detecting an altered level of expression of the reporter molecule in the presence of the one or more test compounds compared to in the absence of the one or more test compounds, thereby identifying the one or more test compounds as altering binding of RelB Rel homology domain (RelB RHD) with a RelBκB sequence.

A "reporter molecule" as used herein refers to RNA and/or polypeptide, *etc.* which is detectable in any detection system, including, but not limited to enzyme (*e.g.*, ELISA, as well as enzyme-based histochemical assays), fluorescent, radioactive, and luminescent systems. Exemplary reporter genes include, for example, luciferase, green fluorescent protein (GFP) gene, *E. coli* β-galactosidase gene, human placental alkaline phosphatase gene, horseradish peroxidase, and chloramphenicol acetyltransferase gene.

The term "level of expression" refers to the quantity of RNA and/or protein that is produced following transcription of a DNA sequence that encodes the RNA and/or protein. Methods for determining the level of expression of proteins are known in the art such as Enzyme Linked Immunosorbent Assay (ELISA) as described herein, immunofluorescence assays wherein the transfected cells are incubated with a first antibody that is specific for the expressed protein and flouresecently labeled second antibody that is specific for the immunoglobulin of the first antibody, followed by observation of immunofluorescence under the microscope, and detecting the activity of the protein (*e.g.*, β-glucuronidase encoded by the *uid A* gene). Methods for determining the level of expression of RNA are known in the art such as Northern blots.

In one embodiment, the nucleotide sequence comprising the RelBκB sequences of the invention are operably linked to the nucleotide sequence encoding the reporter molecule in an expression vector. Expression vectors are exemplified by, but not limited to, plasmid, phagemid, shuttle vector, cosmid, and virus.

The expression vectors of the invention may be introduced into cells using techniques well known in the art. The term "introducing" a nucleic acid sequence into a cell refers to the introduction of the nucleic acid sequence into a target cell to produce a transformed cell. Methods of introducing nucleic acid sequences into cells are well known

in the art. For example, where the nucleic acid sequence is a plasmid or naked piece of linear DNA, the sequence may be "transfected" into the cell using, for example, calcium phosphate-DNA co-precipitation, DEAE-dextran-mediated transfection, polybrene-mediated transfection, electroporation, microinjection, liposome fusion, lipofection, protoplast fusion, and biolistics. Alternatively, where the nucleic acid sequence is encapsidated into a viral particle, the sequence may be introduced into a cell by "infecting" the cell with the virus.

Transformation of a cell may be stable or transient. The terms "transient transformation" and "transiently transformed" refer to the introduction of one or more nucleotide sequences of interest into a cell in the absence of integration of the nucleotide sequence of interest into the host cell's genome. Transient transformation may be detected by, for example, enzyme-linked immunosorbent assay (ELISA) which detects the presence of a polypeptide encoded by one or more of the nucleotide sequences of interest. Alternatively, transient transformation may be detected by detecting the activity of the protein (*e.g.*, β-glucuronidase) encoded by the nucleotide sequence of interest. The term "transient transformant" refer to a cell which has transiently incorporated one or more nucleotide sequences of interest. Transient transformation with the invention's vectors may be desirable in, for example, cell biology or cell cycle investigations which require efficient gene transfer.

In contrast, the terms "stable transformation" and "stably transformed" refer to the introduction and integration of one or more nucleotide sequence of interest into the genome of a cell. Thus, a "stable transformant" is distinguished from a transient transformant in that, whereas genomic DNA from the stable transformant contains one or more nucleotide sequences of interest, genomic DNA from the transient transformant does not contain the nucleotide sequence of interest. Stable transformation of a cell may be detected by Southern blot hybridization of genomic DNA of the cell with nucleic acid sequences which are capable of binding to one or more of the nucleotide sequences of interest. Alternatively, stable transformation of a cell may also be detected by the polymerase chain reaction of genomic DNA of the cell to amplify the nucleotide sequence of interest.

One of skill in the art appreciates that there are several cell type suitable for transformation with expression vectors containing the invention's sequences, such as eukaryotic cells (*e.g.*, yeast, insect, and mammalian cells) and prokaryotic cells (*e.g.*, bacterial cells such as *E. coli*, and viruses).

In one embodiment, it may be desirable to use a control sample containing IKKα having reduced kinase activity (including non-activatable IKKα) compared to wild-type IKKα. Thus, in one embodiment the method further comprises: c) contacting the isolated nucleotide sequence, in the presence of the one or more test compounds, with one or more compositions comprising a polypeptide that comprises RelB RHD, as exemplified by SEQ ID NO:62, wherein the composition is chosen from cell extract, cytoplasmic extract, and nuclear extract, wherein the composition is isolated from a mammalian cell comprising non-activatable IKKα, and wherein the mammalian cell is treated with one or more IKKα activators chosen from lymphotoxin B (LTβ), B cell activating factor belonging to the TNF family (BAFF), anti-LTβR antibody, and CD40 ligand (CD40L); and d) detecting unaltered binding of the isolated nucleotide sequence with RelB RHD, as exemplified by SEQ ID NO:62, in the presence and absence of the one or more test compounds.

In another embodiment, it may be desirable to use RelA:p50 as a control. Thus in one embodiment, the method further comprises detecting unaltered binding of the isolated nucleotide sequence to one or more proteins chosen from a polypeptide comprising one or more of RelA RHD, RelA, p50, and RelA:p50, in the presence and absence of the one or more test compounds.

In a further embodiment, RelA:p52 may be used as control. Thus, the method further comprises detecting unaltered binding of the isolated nucleotide sequence to one or more proteins chosen from a polypeptide comprising one or more of RelA RHD, RelA, p52, and RelA:p52, in the presence and absence of the one or more test compounds.

Where RelB:p50 is used a control, the method may further comprise detecting unaltered binding of the isolated nucleotide sequence to one or more proteins chosen from a polypeptide comprising one or more of RelB RHD, as exemplified by SEQ ID NO:62, RelB, p50, and RelB:p50, in the presence and absence of the one or more test compounds.

Alternatively, one control involves using the consensus-κB sequence by detecting unaltered binding of an isolated nucleotide sequence comprising the consensus-κB sequence 5'-GGGACTTTCC-3' (SEQ ID NO:58) to a polypeptide comprising one or more of RelB RHD, as exemplified by SEQ ID NO:62, and RelB in the presence of the one or more test compounds.

In one embodiment, the method further comprising identifying the one or more test compounds as altering IKKα cellular activity and/or as altering symptoms associated with IKKα related pathology.

J. Detection of specific binding, and screening test compounds, using optical affinity biosensor system assays

The level of specific binding of the isolated nucleotide sequence that contains the invention's RelBκB sequences (*e.g.*, SEQ ID NO:57) with the protein RelB RHD, as exemplified by SEQ ID NO:62, may be determined using optical affinity biosensor system (OABS) assays. The use of optical affinity biosensor systems is known in the art, for example U.S. Patent No. 6,333,153. For example, in an OABS system such as the IAsysTM system (Affinity Sensors, Cambridge, United Kingdom), binding and dissociation events can be detected as one molecule in solution binds to or dissociates from another molecule immobilized on a detector surface of the system. Thus, an OABS may be used to detect specific binding between invention's RelBκB sequences and one or more proteins, such as RelB:p52, RelB, and RelB RHD, as exemplified by SEQ ID NO:62, by immobilizing either the protein or the target DNA on the detector surface of the OABS.

K. Methods for expressing a nucleic acid sequences of interest

The invention also provides methods for inducing expression of a nucleotide sequence of interest by placing this sequence under the regulatory control of the invention RelBκB sequences (*e.g.*, SEQ ID NO:57), and inducing expression by making available proteins that specifically bind to the invention's RelBκB sequences, such as RelB:p52. These methods are useful where it is desirable to have inducible gene expression in response to a stimulus, where constitutive gene expression is less desirable than inducible gene expression. Alternatively, these methods are useful to induce expression of genes which, in their wild type form, are under transcriptional control of the invention's sequences, such as genes whose expression is altered by IKKα activity.

In one embodiment, the invention provides a method for expression of a nucleic acid sequence of interest, comprising: a) providing: i) a cell comprising an isolated nucleotide sequence comprising invention RelBκB sequences (*e.g.*, SEQ ID NO:57), wherein the invention RelBκB sequence is operably linked to the nucleic acid sequence of interest; and ii) a polypeptide comprising RelB Rel homology domain (RelB RHD, as exemplified by SEQ ID NO:62); and b) contacting the cell with the polypeptide such that the RelB RHD, as exemplified by SEQ ID NO:62, specifically binds with invention RelBκB sequences (*e.g.*, SEQ ID NO:57), and the nucleic acid sequence of interest is expressed.

Nucleotide sequences that may be employed in the invention's methods include, for example, genes whose expression may be altered (increased and/or decreased) by IKKα kinase activity, such as genes encoding cytokines such interferon-β (IFN-β), interleukin-1 (IL-1), interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-12 (IL-12), LTα, and LTβ; chemokines such as IL-8, MIP-1α, MCP1, RANTES, and eotaxin; growth factors such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and vascular endothelial growth factor (VEGF); adhesion molecules such as ICAM, VCAM, and E-selectin, acute phase proteins such as SAA; inducible effector enzymes such as iNOS and COX-2; Igκ, CD40 ligand (CD40L), G1 cylcins, cell-cycle regulators such as cyclin D1 and cyclin D2; regulators of apoptosis and cell proliferation such as BCL-X_L, cIAP-1, c-IAP-2, A1/BFL1, Fas ligand, c-myc, and cyclin D1; caspase-8/FADD (FAS-associated death domain)-like IL-1β-converting enzyme (FLICE) inhibitory protein (c-FLIP); several metalloproteinases (MMPs), *i.e.*, proteolytic enzymes that promote tumor invasion of surrounding tissue; HTLV-I trans-activator (tax); evolutionarily conserved antimicrobial peptides such as β defensins; molecules involved in the adaptive immune response such as MHC proteins, costimulatory molecules such as B7.1.

Additional nucleotide sequences that may be employed in the invention's methods include, for example, genes encoding a "protein of interest," as described *supra*.

L. Methods of altering symptoms of diseases associated with IKKα pathology

The invention also provides methods for altering symptoms of IKKα related pathology comprising administering to a mammalian subject one or more compounds that alters binding of RelB Rel homology domain (RelB RHD) with a RelBκB sequence, wherein the one or more compound is identified according to any of the invention's methods. In one embodiment, the invention methods further comprise observing altered symptoms of the IKKα related pathology, wherein the symptoms are reduced or increased. These methods are useful in, for example, identifying compounds (e.g. environmental, chemical, natural occurring, man-made, *etc.*) that may be implicated in causation and/or exacerbation of IKKα related pathology. Additionally, these methods are useful to identify therapeutic compound that reduce symptoms associated with IKKα related pathology.

The terms "pathology," "disease" and "pathological condition" are used interchangeably to refer to a state, signs, and/or symptoms that are associated with any impairment, interruption, cessation, or disorder of the normal state of a living animal or of any of its organs or tissues that interrupts or modifies the performance of normal functions, and may be a response to environmental factors (such as malnutrition, industrial hazards, or climate), to specific infective agents (such as worms, bacteria, or viruses), to inherent defect of the organism (such as various genetic anomalies, or to combinations of these and other factors. The term "disease" includes responses to injuries, especially if such responses are excessive, produce symptoms that excessively interfere with normal activities of an individual, and/or the tissue does not heal normally (where excessive is characterized as the degree of interference, or the length of the interference).

In particular, the term "IKKα related pathology" refers to pathologies that are associated with altered levels of expression of IKKα, altered levels of kinase activity of IKKα, and/or altered levels of binding of the RelB Rel homology domain (RHD) that is exemplified by SEQ ID NO:62, with a cell's RelBκB sequences (such as SEQ ID NO:57). In one embodiment, IKKα related pathologies may involve overproliferation and/or underproliferation of cells, such as B cells, mammary epithelial cells, cells involved in inflammatory disease, epithelial cells involved in disease such as cancer, and cells involved in infection by microorganisms.

For example, IKKα related pathologies involving overproliferation and/or underproliferation of B cells are exemplified, but not limited to, spleen disorganization, lymphomas (such as diffuse large B-cell lymphomas (DBCLs) which include germinal-center-like and B-cell-like, Hodgkin's B-cell lymphoma, non-Hodgkin's B-cell lymphomas, B-cell lymphomas, virus-induced lymphomas, Birkett's lymphoma, mucosa-associated lymphoid tissue (MALT) lymphomas), leukemia (such as chronic lymphocytic leukaemia (CLL), B-cell lymphocytic leukaemia (B-CLL), virus-induced leukaemias), lymphoid hyperplasia, splenomegaly.

IKKα related pathologies involving overproliferation and/or underproliferation of mammary epithelial cells are exemplified by defects in lactation, defects in alveolar development such as during pregnancy, lactation, and involution, and mammary cancer.

IKKα related pathologies may also involve overproliferation and/or underproliferation of cells involved in inflammatory disease such as, without limitation, sepsis, septic shock, endotoxic shock, inflammatory bowel disease (IBD) such as Crohn's

disease and ulcerative colitis, multiple sclerosis, inflammatory diseases involving acute or chronic inflammation of bone and/or cartilage in a joint, anaphylactic reaction, nephritis, asthma, conjunctivitis, inflammatory gum disease, systemic lupus erythematosus, insulin dependent diabetes mellitus, pulmonary sarcoidosis, ocular inflammation, allergy, emphysema, ischemia-reperfusion injury, fibromyalagia, an inflammatory cutaneous disease selected from psoriasis and dermatitis, or an arthritis selected from rheumatoid arthritis, gouty arthritis, juvenile rheumatoid arthritis, and osteoarthritis.

IKKα related pathologies may also involve overproliferation and/or underproliferation of cells infected by a microorganism. The terms "infection by a microorganism" and "microbial infection" are used interchangeably to refer to the undesirable presence of a microorganism (such as bacteria, fungi, protozoa and/or viruses) in a subject. Bacterial and/protozoal infections and disorders related to such infections in humans include the following: pneumonia, otitis media, sinusitis, bronchitis, tonsillitis, and mastoiditis related to infection by *Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis, Staphylococcus aureus,* or *Peptostreptococcus* spp.; pharynigitis, rheumatic fever, and glomerulonephritis related to infection by *Streptococcus pyogenes,* Groups C and G streptococci, *Clostridium diptheriae,* or *Actinobacillus haemolyticum*; respiratory tract infections related to infection by *Mycoplasma pneumoniae, Legionella pneumophila, Streptococcus pneumoniae, Haemophilus influenzae,* or *Chlamydia pneumoniae;* uncomplicated skin and soft tissue infections, abscesses and osteomyelitis, and puerperal fever related to infection by *Staphylococcus* aureus, coagulase-positive staphylococci (*i.e., S. epidermidis, S. hemolyticus, etc.*), *Streptococcus pyogenes, Streptococcus agalactiae,* Streptococcal groups C-F (minute-colony streptococci), *Viridans streptococci, Corynebacterium minutissimum, Clostridium* spp., or *Bartonella henselae;* uncomplicated acute urinary tract infections related to infection by *Staphylococcus saprophyticus* or *Enterococcus* spp.; urethritis and cervicitis; and sexually transmitted diseases related to infection by *Chlamydia trachomatis, Haemophilus ducreyi, Treponema pallidum, Ureaplasma urealyticum,* or *Neiserria gonorrheae*; toxin diseases related to infection by *S. aureus* (food poisoning and Toxic shock syndrome), or Groups A, B, and C streptococci; ulcers related to infection by *Helicobacter pylori*; systemic febrile syndromes related to infection by *Borrelia recurrentis*; Lyme disease related to infection by *Borrelia burgdorferi;* conjunctivitis, keratitis, and dacrocystitis related to infection by *Chiamydia trachomatis, Neisseria gonorrhoeae, S. aureus, S. pneumoniae, S. pyogenes, H. influenzae,*

or *Listeria* spp.; disseminated Mycobacterium avium complex (MAC) disease related to infection by *Mycobacterium avium*, or *Mycobacterium intracellulare*; gastroenteritis related to infection by *Campylobacter jejuni*; intestinal protozoa related to infection by *Cryptosporidium* spp.; odontogenic infection related to infection by *Viridans streptococci*; persistent cough related to infection by *Bordetella pertussis;* gas gangrene related to infection by *Clostridium perfringens* or *Bacteroides* spp.; and atherosclerosis related to infection by *Helicobacter pylori* or *Chlamydia pneumoniae,* and gastrointestinal infections related to the protozoa *Entameba histolytica, Cryptosporidium parvum, Giardia lamblia,* and *amoebae.*

Frequently seen parasitic protozoal diseases include malaria, caused by the four *Plasmodium* species: *P. falciparum, P. vivax, P. ovale* and *P. malariae*, toxoplasmosis, caused by *Toxoplasma gondii,* leishmaniasis, caused by *Leishmania* species such as *Leishmania donovani,* and Chagas' disease (American trypanosomiasis), caused by *Trypanosoma cruzi.*

Fungal infections of humans include candidiasis, aspergillosis, mucormycosis, cryptococcosis, ringworms (caused by *Tinea corpis* and *Tinia capitis*), athlete's foot (caused by *Tinea pedis*), nail infections (caused by onychomycosis), and "jock itch" (caused by *Tinea cruris*).

Viral infections of humans include infections with polyomavirus, papillomavirus, T-cell leukemia virus), herpes simplex virus, adenovirus, Rous sarcoma virus, cytomegalovirus, retroviruses such as enteroviruses, Epstein Barr virus (EBV) implicated in Burkitt's and Hodgkins lymphomas, as well as B-cell lymphomas in immunocompromised hosts, HIV virus, hepatitis B virus, pseudorabies virus, papilloma virus.

IKKα related pathologies may also involve overproliferation and/or underproliferation of epithelial cell, such as epithelial cell cancer. The terms "cancer" refers to a neoplasm which contains at least one cancer cell. A "cancer cell" refers to a cell undergoing early, intermediate or advanced stages of multi-step neoplastic progression as previously described (H.C. Pitot (1978) in "Fundamentals of Oncology," Marcel Dekker (Ed.), New York pp 15-28), including pre-neoplastic cell (*i.e.*, hyperplastic cell and dysplastic cell) and neoplastic cell. The term "cancer" is used herein to refer to a neoplasm, which may or may not be metastatic. Exemplary cancers within the scope of the invention include carcinomas such as breast cancer, gastric cancer, lung cancer, prostate cancer, cervical cancer, pancreatic cancer, colon cancer, colorectal cancer, ovarian cancer; stomach

cancer, esophagus cancer, mouth cancer, tongue cancer, gum cancer, skin cancer (e.g., melanoma, basal cell carcinoma, Kaposi's sarcoma, *etc.*), muscle cancer, heart cancer, liver cancer, bronchial cancer, cartilage cancer, bone cancer, testis cancer, kidney cancer, endometrium cancer, uterus cancer, bladder cancer, bone marrow cancer, lymphoma cancer, spleen cancer, thymus cancer, thyroid cancer, brain cancer, neuron cancer, mesothelioma, gall bladder cancer, ocular cancer (*e.g.*, cancer of the cornea, cancer of uvea, cancer of the choroids, cancer of the macula, vitreous humor cancer, *etc.*), joint cancer (such as synovium cancer), glioblastoma, lymphoma, leukemia, and hereditary non-polyposis cancer (HNPC), colitis-associated cancer. Cancers are further exemplified by sarcomas (such as osteosarcoma and Kaposi's sarcoma).

Other IKKα related pathologies include, for example, multiple myeloma, T-cell lymphomas, sporadic adenomatous polyps, hereditary familial adenomatous polyposis (FAP).

The compounds identified in accordance with the invention's methods may be administered in a pharmaceutically effective amount. As used herein, the actual amount encompassed by the term "pharmaceutically effective amount" will depend on the route of administration, the type of subject being treated, and the physical characteristics of the specific subject under consideration. These factors and their relationship to determining this amount are well known to skilled practitioners in the medical, veterinary, and other related arts. This amount and the method of administration can be tailored to achieve optimal efficacy but will depend on such factors as weight, diet, concurrent medication and other factors which those skilled in the art will recognize.

The dosage amount and frequency are selected to create an effective level of the compound without substantially harmful effects. A pharmaceutically effective amount may be determined using *in vitro* and *in vivo* assays known in the art.

Methods of administering a pharmaceutically effective amount of the invention's compounds are well known in the art and include, without limitation, administration in parenteral, oral, intraperitoneal, intranasal, topical, sublingual, rectal, and vaginal forms. Parenteral routes of administration include, for example, subcutaneous, intravenous, intramuscular, intrastemal injection, and infusion routes.

The compounds may be administered before, concomitantly with, and/or after manifestation of one or more symptoms of a disease or condition. Also, the compounds

may be administered before, concomitantly with, and/or after administration of another type of drug or therapeutic procedure (*e.g.*, surgery).

Pharmaceutical compositions preferably comprise one or more compounds of identified in accordance with the present invention, that are associated with one or more pharmaceutically acceptable carrier, diluent or excipient. In preparing such compositions, the active ingredients are usually mixed with or diluted by an excipient or enclosed within such a carrier which can be in the form of a capsule or sachet in which the coating may be gelatin, sugar, shellac, and other enteric coating agents. When the excipient serves as a diluent, it may be a solid, semi-solid, or liquid material which acts as a vehicle, carrier, or medium for the active ingredient. Thus, the compositions can be in the form of tablets, pills, powders, elixirs, suspensions, emulsions, solutions, syrups, soft and hard gelatin capsules, suppositories, sterile injectable solutions and sterile packaged powders. Examples of suitable excipients include lactose, dextrose, sucrose, sorbitol, mannitol, starch, gum acacia, calcium silicate, microcrystalline cellulose, polyvinylpyrrolidone, cellulose, water, syrup, and methyl cellulose.

Pharmaceutically acceptable carriers are known in the art such as those described in, for example, Remingtons Pharmaceutical Sciences, Mack Publishing Co. (A. R. Gennaro edit. 1985). Exemplary pharmaceutically acceptable carriers are sterile saline, phosphate-buffered saline at physiological pH, polyethylene glycols, polypropylene copolymers, and water soluble gels.

Other compounds that may be included with the invention's compositions include, for example, diluents, fillers, salts, buffers, preservatives (*e.g.*, sodium benzoate, sorbic acid and esters of p-hydroxybenzoic acid), stabilizers, dyes, antioxidants, flavoring agents, lubricating agents (such as talc, magnesium stearate and mineral oil), wetting agents, emulsifying and suspending agents, preserving agents such as methyl- and propylhydroxybenzoates, sweetening agents and/or flavoring agents.

The pharmaceutically acceptable carriers may be liquid, with the compositions being, for example, an oral syrup or injectable liquid. Compositions in solid or liquid form may include an agent which binds to the active component(s) and thereby assists in the delivery of the active components. Suitable agents which may act in this capacity include a monoclonal or polyclonal antibody, a protein or a liposome.

Alternatively, the pharmaceutical composition of the present invention may consist of gaseous dosage units, *e.g.*, it may be in the form of an aerosol useful in, for example,

inhalatory administration. The term "aerosol" is used to denote a variety of systems ranging from those of colloidal nature to systems consisting of pressurized packages. Delivery may be by a liquefied or compressed gas or by a suitable pump system which dispenses the active ingredients. Aerosols of compounds of the invention may be delivered in single phase, bi-phasic, or tri-phasic systems in order to deliver the active ingredient(s). Delivery of the aerosol includes the necessary container, activators, valves, subcontainers, spacers and the like, which together may form a kit. Preferred aerosols may be determined by one skilled in the art, without undue experimentation.

When intended for oral administration, the composition is preferably in either solid or liquid form, where semi-solid, semi-liquid, suspension and gel forms are included within the forms considered herein as either solid or liquid.

As a solid composition for oral administration, the composition may be formulated into a powder, granule, compressed tablet, pill, capsule, chewing gum, wafer or the like form. Such a solid composition will typically contain one or more inert diluents or edible carriers. In addition, one or more of the following adjuvants may be present: binders such as carboxymethylcellulose, ethyl cellulose, microcrystalline cellulose, or gelatin; excipients such as starch, lactose or dextrins, disintegrating agents such as alginic acid, sodium alginate, Primogel, corn starch and the like; lubricants such as magnesium stearate or Sterotex; glidants such as colloidal silicon dioxide; sweetening agents such as sucrose or saccharin, a flavoring agent such as peppermint, methyl salicylate or orange flavoring, and a coloring agent.

The pharmaceutical composition may be intended for topical administration, in which case the carrier may suitably comprise a solution, emulsion, ointment or gel base. The base, for example, may comprise one or more of the following: petrolatum, lanolin, polyethylene glycols, beeswax, mineral oil, diluents such as water and alcohol, and emulsifiers and stabilizers. Thickening agents may be present in a pharmaceutical composition for topical administration. If intended for transdermal administration, the composition may include a transdermal patch or iontophoresis device.

The composition may be intended for rectal administration, for example in the form of a suppository which will melt in the rectum and release the drug. The composition for rectal administration may contain an oleaginous base as a suitable nonirritating excipient. Such bases include, without limitation, lanolin, cocoa butter and polyethylene glycol.

When the composition is in the form of a capsule, *e.g.*, a gelatin capsule, it may contain, in addition to materials of the above type, a liquid carrier such as polyethylene glycol, cyclodextrin or a fatty oil.

The composition may be in the form of a liquid, *e.g.*, an elixir, syrup, solution, emulsion or suspension. The liquid may be for oral administration or for delivery by injection, as two examples. When intended for oral administration, preferred compositions contain, in addition to the invention's compounds, one or more of a sweetening agent, preservatives, dye/colorant and flavor enhancer. In a composition intended to be administered by injection, one or more of a surfactant, preservative, wetting agent, dispersing agent, suspending agent, buffer, stabilizer and isotonic agent may be included.

The liquid pharmaceutical compositions of the invention, whether they be solutions, suspensions or other like form, may include one or more of the following adjuvants: sterile diluents such as water for injection, saline solution, preferably physiological saline, Ringer's solution, isotonic sodium chloride, fixed oils such as synthetic mono or digylcerides which may serve as the solvent or suspending medium, polyethylene glycols, glycerin, cyclodextrin, propylene glycol or other solvents; antibacterial agents such as benzyl alcohol or methyl paraben; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic. Physiological saline is a preferred adjuvant. An injectable pharmaceutical composition is preferably sterile.

The pharmaceutical compositions may be prepared by methodology well known in the pharmaceutical art. A composition intended to be administered by injection can be prepared by combining the compound of any one of Formulae A-E with water so as to form a solution. A surfactant may be added to facilitate the formation of a homogeneous solution or suspension. Surfactants are compounds that non-covalently interact with the compound of any one of Formulae A-E so as to facilitate dissolution or homogeneous suspension of the active compound in the aqueous delivery system.

M. Kits

The invention also provides kits comprising an isolated nucleotide sequence comprising one or more of the invention's RelBκB sequences. In one embodiment, the kit

further comprises instructions for binding the isolated nucleotide sequence with a polypeptide comprising RelB RHD, as exemplified by SEQ ID NO:62. The kits are useful in any of the invention's methods.

As used herein, the term "kit" is used in reference to a combination of reagents and other materials. It is contemplated that the kit may include reagents such as buffering agents, nucleic acid stabilizing reagents, protein stabilizing reagents, signal producing systems (*e.g.*, florescence generating systems as Fret systems), antibodies, control proteins, control nucleic acid sequences, as well as testing containers (*e.g.*, microtiter plates, *etc.*). It is not intended that the term "kit" be limited to a particular combination of reagents and/or other materials. In one embodiment, the kit further comprises instructions for using the reagents. The test kit may be packaged in any suitable manner, typically with the elements in a single container or various containers as necessary along with a sheet of instructions for carrying out the test. In some embodiments, the kits also preferably include a positive control sample. Kits may be produced in a variety of ways known in the art.

O. Additional Considerations

Two distinct pathways leading to selective activation of RelA:p50 and RelB:p52 dimers, dependent on IKKβ or IKKα, respectively, were identified (Ghosh *et al.* (2002) Cell, 109, S81-96). Each pathway has distinct biological functions (Chen *et al.* (2003) Nat Med, 9, 575-581; Li *et al.* (1999) Science, 284, 321-325; Senftleben *et al.* (2001) Science, 293, 1495-1499), that could be mediated in part through selective gene activation (Dejardin *et al.* (2002) Immunity, 17, 525-535). How this occurs was previously unknown. We now show in two exemplary cell types, splenic stromal cells and BMDC, that IKKα is required for induction of four genes encoding chemokines critical for spleen organogenesis and maintenance of tissue microarchitecture, because these genes are selectively recognized by RelB-containing dimers, most likely RelB:p52. These genes are preferentially activated by engagement of LTβR and are weakly induced by TNFα. Whereas the TNFα response is IKKα-independent, the response to LTβR engagement is strictly IKKα-dependent. The

latter requires two events. First, RelB:p52 dimers have to enter the nucleus, a process dependent on IKKα-mediated p100 processing (Dejardin *et al.* (2002) Immunity, 17, 525-535; Yilmaz *et al.* (2003) Embo J, 22, 121-130). Second, RelB:p52 dimers are selectively recruited to the IKKα-dependent gene promoter. The selective recruitment of RelB to the *Blc* and the *Elc* promoters is likely to depend on a novel κB site, whose consensus sequence (Fig 30D) is distinct from that of the classical κB site. Unlike the classical site, the novel site is preferentially recognized by RelB:p52 dimers. This unique sequence specificity is entirely consistent with sequence differences between the DNA binding loops of RelA and RelB, but was previously unknown (Ghosh *et al.* (1995) Nature, 373, 303-310). It is entirely possible, however, that additional factors may contribute to selective IKKα-dependent gene activation and that IKKα may also be responsible in certain cell types for activation of the canonical NF-κB pathway (Cao *et al.* (2001) Cell, 107, 763-775) or for potentiating its ability to activate transcription (Anest *et al.* (2003) Nature, 423, 659-663; Israel *et al.* (2003) Nature, 423, 596-597; Yamamoto *et al.* (2003) Nature, 423, 655-659). Nonetheless, an important mechanism responsible for selective gene activation through the IKKα-dependent alternative NF-κB signaling pathway is based on specific recruitment of RelB:p52 dimers to target gene promoters. Sites similar to the RelB:p52 selective κB site were detected in the 5' regulatory region of three other genes, whose expression was found to be IKKα-dependent (Fig 30D).

It is the inventor's view that IKKβ-mediated NF-κB signaling is responsible for rapid responses to infection and injury that require recruitment of immune cells out of lymphoid organs to sites of infection. This response depends on pro-inflammatory chemokines, such as MIP-1, MCP-1 and RANTES, which are induced by the canonical NF-κB signaling pathway (Alcamo *et al.* (2001) J Immunol, 167, 1592-1600). The arrival of

antigens to secondary lymphoid tissues from distal sites of infection and their processing, presentation and recognition require coordinated activity of DC, macrophages, T cells and B cells, whose recruitment to secondary lymphoid organs depends on IKKα-regulated organogenic chemokines. Premature expression of such chemokines would compromise the immediate anti-microbial response as it may abort the emigration of immune cells to the periphery. It is, therefore, logical that expression of organogenic chemokines would not be induced through the canonical NF-κB signaling pathway. Consistent with its delayed function in adaptive immunity, activation of the alternative NF-κB signaling pathway is slower than the canonical NF-κB signaling pathway and seems to depend on prior activation of the latter (Dejardin *et al.* (2002) Immunity, 17, 525-535). The dependence of the two pathways on distinct but related protein kinases and transcription factors allows for both functional integration and kinetic separation.

EXPERIMENTAL

The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

EXAMPLE 1

Materials And Methods

The following is a brief description of exemplary materials and methods that may be used in the invention's methods.

A. Primary cell cultures

Primary stromal cell cultures were established from spleens of wild type (WT) and *Ikkα*$^{AA/AA}$ mice as described (Skibinski *et al.* (1998) Eur J Immunol 28, 3940-3948). Briefly, spleens were gently ground and released cells cultured in DMEM supplemented with heat-inactivated FCS (Invitrogen, Carlsbad, CA). After one week, non-adherent cells were removed, adherent cells were washed twice with PBS and cultured one more week in

DMEM/FCS. Absence of contaminating myeloid and lymphoid cells was verified by flow cytometry (FACSCalibur, Becton Dickinson) using antibodies to B220, TCRβ and MAC-1 (all from BD Pharmingen, San Diego, CA). Stromal cells were uniformly positive for ICAM-1 (clone BBIG-I1 R&D Systems, Minneapolis, Mn).

B. Adoptive transfers

Bone marrow cells (3-4 x 10^6 cells per mouse) were isolated from femurs of WT or *Ikkα*$^{AA/AA}$ mice and injected intravenously via the tail vein into sublethally or lethally irradiated recipients. Mice were H-2 matched and, in the case of *Ikkα*$^{AA/AA}$, were from the F3-F5 backcross to C57Bl/6. Generation of chimeric mice from *Ikkα*$^{-/-}$ fetal liver cells was as described (Senftleben *et al.* (2001a) Science 293, 1495-1499). Mice were provided antibiotics in drinking water and sacrificed 6-8 weeks post reconstitution. Splenocytes were prepared by gentle mincing of spleens between frosted glass slides; 10^7 cells were introduced into sublethally irradiated (750 rad) *Rag2*$^{-/-}$ mice (C57Bl/6 Taconic, Germantown, NY) by tail vein injection and sacrificed 10 days post-transfer. B-cells were prepared by depletion with anti-CD43 magnetic beads (MACS, Auburn, CA) resulting in 92-95% purity. 10^7 purified B-cells were injected i.v into B-cell deficient *mu*MT mice (Jackson Laboratory, Bar Harbor, ME). Recipient mice were sacrificed 15-20 days post-transfer. Some mice were immunized intraperitoneally (i.p.) with SRBC (Colorado Serum Company, Denver, Co) 7 days prior to sacrifice (Poljak *et al.* (1999) J Immunol 163, 6581-6588).

C. Immunohistochemical analysis

Cryosections (8 – 10 μM) of spleen were prepared, dried and fixed with acetone before immunohistochemical analysis (Poljak *et al.* (1999) J Immunol 163, 6581-6588; Weih *et al.* (2001) J Immunol 167, 1909-1919). Staining reagents were: ER-TR9 (RDI, Flanders NJ), FDC-M2 (ImmunoKontact, UK), BM-8-bio (RDI), ICAM-1 (Santa-Cruz Biologicals, CA), MOMA-1 (FITC Calbiochem), MAdCAM (clone MECA-3670), CD11c-bio (clone HL-3), B220, and CD35-bio (clone 8C12) (all from BD Pharmingen). Primary antibodies were revealed using species-specific secondary reagents. Sections were viewed by immunofluorescence microscopy (HM505E Microm Inc, Walldorf, Germany) and images captured with a digital camera (Nikon E800 Scope with Spot Diagnostics Digital Camera, A.G. Heinze Inc., Lake Forest, CA).

D. Electrophoretic mobility shift assay (EMSA) and immunoblotting

Nuclear and cytoplasmic extracts were prepared and analyzed as previously described for the levels of NF-κB subunits and DNA binding activity (Bonizzi *et al.* (1999) Mol Cell Biol 19, 1950-1960; Mercurio *et al.* (1993) Genes Dev 7, 705-718; Senftleben *et al.* (2001a) Science 293, 1495-1499). Recombinant NF-κB subunits were produced in *E.coli* and purified as described (Chen *et al.*, 1999).

E. Analysis of gene expression

RNA was extracted from WT and *Ikkα*$^{AA/AA}$ stromal cells after stimulation with an agonistic anti-LTβR antibody, or from total splenocytes of naïve and immunized WT and *Ikkα*$^{AA/AA}$ mice (Cao *et al.* (2001) Cell 107, 763-775). Real-time PCR™ was performed using a PE Biosystems 5700 thermocycler following the SyBr Green™ detection protocol as outlined by the manufacturer. Briefly, 12 ng of total cDNA, 50 nM of each primer and 1x SyBr Green™ mix were used in a total volume of 25 µl. All values were standardized to that of cyclophilin mRNA. Primer sequences used for real-time PCR™ were as follows: IkB (5'-CCAGAACAACCTGCAGCAGAC-3' (SEQ ID NO:21) and 5'-GCTCAGGATCACAGCCAGCTT-3' (SEQ ID NO:22)), ELC (5'-CATCTGAGCGATTCCAGTCA-3' (SEQ ID NO:23), 5'-ACTGTGTGCGCAAGAATCTG-3' (SEQ ID NO:24)), SLC (5'-GAGTGTCTCCCAGGGAATGA-3' (SEQ ID NO:25), 5'-CTTGGGACCTGAGTGACCCT-3' (SEQ ID NO:26)), BLC (5'-CCATTTGGCACGAGGATTCAC-3' (SEQ ID NO:27), 5'-ATGAGGCTCAGCACAGCAAC-3' (SEQ ID NO:28)), CXCR7 (5'-GAGAGACAAGAACCAAAAGCAC-3' (SEQ ID NO:29), 5'-GGGAAGAATTAGGAGGAAAAGG-3' (SEQ ID NO:30)), CXCR5 (5'-ACTACCCACTAACCCTGGAC-3' (SEQ ID NO:31), 5'-AGGTGATGTGGATGGAGAGGAG-3' (SEQ ID NO:32)), BAFF (5'-AGCTCCAGGAGAAGGCAACTC-3' (SEQ ID NO:33), 5'-ACGGCACGCTTATTTCTGCT-3' (SEQ ID NO:34)), LTα (5'-CAGCAAGCAGAACTCACTGC-3' (SEQ ID NO:35), 5'-AAGAGAAGCCATGTCGGAGA-3' (SEQ ID NO:36)), LTβ (5'-TACACCAGATCCAGGGGTTC-3' (SEQ ID NO:37), 5'-

GAGCTCAGGGTTGAGGTCAG-3' (SEQ ID NO:38)), LTβR (5'-CGGGACACTTCCAGAACACT-3' (SEQ ID NO:39), 5'-CCCTGGATCTCACATCTGGT-3' (SEQ ID NO:40)), TNFα (5'-ACAGAAAGCATGATCCGCG-3' (SEQ ID NO:41), 5'-GCCCCCCATCTTTTGGG-3' (SEQ ID NO:42)), VCAM (5'-GACAGCCCACTAAACGCGAA-3' (SEQ ID NO:43), 5'-CAGAACGGACTTGGACCCCT-3' (SEQ ID NO:44)), ICAM-1 (5'-GATCACATTCACGGTGCTG-3' (SEQ ID NO:45), 5'-GAGAAATTGGCTCCGTGGTC-3' (SEQ ID NO:46)).

F. Chromatin immunoprecipitation assays (ChIP)

ChIP assays were carried out as described (Saccani and Natoli (2002) Dynamic changes in histone H3 Lys 9 methylation occurring at tightly regulated inducible inflammatory genes, Genes Dev *16*, 2219-2224). Polyclonal antibodies to p65 (C-20), RelB (C-19) and Pol II (N-19) were from the supplier Santa Cruz. The following sequences of the promoter-specific primers were used: *Blc* +12 to –688 (5'-GACAAATGTATAAATATTTACTGA-3' (SEQ ID NO:47) and 5'-AACCTTTAGCTCGGAGTCTGCAT-3' (SEQ ID NO:48), *Sdf-1* +22 to -678 (5'-TTCGTACCATCCACCCACCCCCAG-3' (SEQ ID NO:49), and 5'-ACCGAGAGTGAAAGTGCGGCAGCG-3' (SEQ ID NO:50)), *Vcam-1* +30 to -640 (5'-GGCATTTAAGACACTTAATTG-3' (SEQ ID NO:51) and 5'-ATAAATCTCTGGCTTTTCCTG-3' (SEQ ID NO:52)), *Iκbα* +20 to –340 (5'-CGCTAAGAGGAACAGCCTAG-3' (SEQ ID NO:53) and 5'-CAGCTGGCTGAAACATGG-3' (SEQ ID NO:54)), and *Tnfα* +20 to -545 (5'-GCTGCTCTGCCTTCAGCCAGC-3' (SEQ ID NO:55) and 5'-TCCACGCTGAGGGAGCTTCT-3' (SEQ ID NO:56)).

G. Construction, expression and purification of an exemplary DNA-binding domain (DBD) of human p52 (residues 1-340 of SEQ ID NO:64) and of murine RelB (residues 1-400 of SEQ ID NO:62)

1. Construction of the p52 DBD *E. coli*E. coli expression vector:

cDNA corresponding to human p52 was amplified by polymerase chain reaction (PCR) using two primers. The 5' primer contained an Nde1 restriction endonuclease (RE) cleavage site and the 3' primer contained the BamH1 RE cleavage site. The PCR product

was cleaned and restricted by Nde1 and BamH1 which is referred to as the insert. An *E. coli*E. coli T7-expression vector (pET11a, Novagen) was also restricted by the same endonuclease pair. The linearized vector and the insert were mixed and ligated together in the presence of DNA ligase, followed by identification of the correct recombinant plasmid.

2. Bacterial Expression of p52 DBD:

The recombinant plasmid was transformed into an *E. coli*E. coli strain, BL21(DE3). A 2 ml LB broth was inoculated with a fresh single colony and was grown for three hours. The small culture was transferred into a 2L broth for large scale protein production. The culture was grown to optical density (O.D.) of approximately 0.4 at 37°C followed by induction with 0.1 mM IPTG. Induction allows the expression of p52 DBD in large amounts. The culture was allowed to grow for an additional 10-12 hours at room temperature.

3. p52 DBD Purification:

Cells were pelleted by centrifugation and the pellet was suspended in buffer A (20 mM Tris 7.5, 50 mM NaCl, 10 mM bME, 0.1 mM EDTA and 0.1 mM PMSF). Suspended cells were sonicated followed by centrifugation to remove cell debris. Clear supernatant was then loaded onto an anion exchange column pre-equilibrated with buffer A. The flow through containing p52 DBD was collected and loaded onto a cation exchange column. The bound protein was eluted by a linear gradient of NaCl at a concentration from 50 mM to 500 mM. Fractions were analyzed by SDS-PAGE and the fractions containing p52 DBD were pooled, concentrated and subjected to size exclusion chromatography. The peak fractions containing the p52 DBD were pooled and concentrated for further use.

4. Construction of RelB DBD *E. coli* expression vector:

Constrction of an exemplary recombinant murine RelB DBD expression vector was done essentially as described above for the p52 DBD expression vector with the exception of the use of pET15b (Novagen) instead of pET11a. This vector expresses RelB DBD as a poly histidine fusion protein.

5. Bacterial Expression of RelB DBD:

Expression of RelB DBD was done essentially as described above for p52 DBD expression.

6. RelB DBD Purification:

RelB DBD was purified using two chromatographic steps. In the first step, the supernatant of cell extract was loaded onto a Ni affinity column which specifically binds to poly-histidine peptide. In this case the poly histine tagged RelB DBD (His-RelB DBD) remained bound to the column. Bound protein was eluted with 250 mM imidazole which competes with histindine for binding to the Ni^{2+} affinity matrix. Eluted His-RelB DBD was concentrated and loaded onto a size exclusion column, Superdex 200 column (Pharmacia). Fractions containing His-RelB DBD were pooled and concentrated.

H. Preparation of an exemplary Rel DBD/p52 DBD heteordimer:

His-RelB DBD and p52 DBD that had been purified as described above were mixed together in 1:1.2 molar ratio (*i.e.*, with excess p52 DBD) and diluted to ~0.4 mg/ml concentration in a denaturing buffer B (7 M urea, 0.5 M NaCl, 20 mM Tris pH 7.5, 5 mM DTT, 10% glycerol). The denatured protein mixture (100 ml total volume that contained 40 mg total protein) was refolded by slowly removing urea by dialysis. The sample was subjected to dialysis against 2 L buffer C (buffer C was the same as buffer B except it lacked urea) for 6 hours. The dialyis step was repeated two more times. After dialysis, the refolded heterodimer was loaded onto the Ni-affinity column. Excess, uncomplexed p52 DBD flows through the column because it has no poly-His tag to bind Ni-affinity matix. The heterodimer was eluted with imidazole. The eluate was concentrated and further purified using size exclusion chromatography (Superdex 200, Pharmacia). Fractions containing the heterodimer were pooled and concentrated. The presence of both proteins was confirmed by standard western blotting.

EXAMPLE 2

Stromal cell-derived chemokine production requires IKKα

Lethally irradiated mice reconstituted with *Ikkα*$^{-/-}$ hematopoietic progenitors revealed a role for IKKα in late B-cell maturation, splenic organization and germinal center (GC) formation (U. Senftleben *et al.*, *Science* 293, 1495-1499 (2001); T. Kaisho *et al.*, *J.*

Exp. Med. 193, 417-426 (2001)). However, embryonic lethality precludes the use of *Ikkα*$^{-/-}$ mice to identify functions for IKKα in other cell types. Homozygous knock-in mice expressing an IKKα variant that cannot be activated (*Ikkα*$^{AA/AA}$ mice) are viable, yet show defective lymphoid organogenesis and GC formation (U. Senftleben *et al.*, *Science* 293, 1495-1499 (2001)). To identify the cells in which IKKα acts to control secondary lymphoid organogenesis, reciprocal bone marrow chimeras were generated between *Ikkα*$^{AA/AA}$ and WT mice. The chimeric mice were challenged with a T-cell dependent antigen, sheep red blood cells (SRBC), and sacrificed 7 days later. Using an antibody against FDC-M2 or CD35, we examined formation of mature follicular dendritic cells (FDC). FDC maturation was impaired in *Ikkα*$^{AA/AA}$ recipients reconstituted with WT bone marrow, whereas a mature FDC network formed in WT recipients reconstituted with *Ikkα*$^{AA/AA}$ bone marrow (Fig 1A). These results suggest that IKKα acts in stromal cells of the spleen to induce their maturation into FDCs.

Another aspect of proper splenic development is segregation of B and T-lymphocytes to the follicles and the peri-arterial lymphatic sheath (PALS), respectively. WT chimeras reconstituted with *Ikkα*$^{AA/AA}$ bone marrow, but not *Ikkα*$^{AA/AA}$ mice reconstituted with WT bone marrow, exhibited normal B- and T- cell segregation (Fig 1B). These results also point to a critical action of IKKα in stromal cells, which control splenic microarchitecture through production of organogenic chemokines that dictate cell migration and positioning (K. M. Ansel, J. G. Cyster, *Curr Opin Immunol* 13, 172-179 (2001)), other than the hematopoietic compartment as previously assumed (U. Senftleben *et al.*, *Science* 293, 1495-1499 (2001); T. Kaisho *et al.*, *J. Exp. Med.* 193, 417-426 (2001)). Critical chemokines for spleen development include ELC and SLC, ligands for the chemokine receptor CCR7, BLC, which binds CXCR5 (R. Forster *et al.*, *Cell* 99, 23-33 (1999); K. M. Ansel *et al.*, *Nature* 406, 309-314 (2000)) and SDF-1, which promotes trafficking of both immature and naïve lymphocytes to lymphoid tissues (C. H. Kim, H. E. Broxmeyer, *J Leukoc Biol* 65, 6-15 (1999)). Previous work revealed that induction of these chemokines in response to engagement of LTβR is defective in *Ikkα*$^{AA/AA}$ mice (E. Dejardin *et al.*, *Immunity* 17, 525-535 (2002)). We extended these observations to SRBC immunized mice (Fig 1C).

EXAMPLE 3

IKKα is required for LTβR–induced RelB:p52 nuclear translocation and chemokine expression in splenic stromal cells and myeloid dendritic cells

The defects described in Example 2 were very similar to those exhibited by mice lacking LTβR (Y. X. Fu, D. D. Chaplin, *Annu Rev Immunol* 17, 399-433 (1999)). The major cell type expressing LTβR in the spleen is the stromal cell. To examine the role of IKKα in LTβR signaling in splenic stromal cells, as well as in bone marrow derived dendritic cells (BMDC), which also express LTβR (J. L. Browning, L. E. French, *J Immunol* 168, 5079-5087 (2002)), we isolated and cultured these cells from WT and *Ikkα*$^{AA/AA}$ mice. Stimulation of WT stromal cells with agonistic anti-LTβR antibody (E. Dejardin *et al.*, *Immunity* 17, 525-535 (2002)) resulted in 4-6-fold induction of BLC, SDF-1, TNFα and VCAM-1 mRNAs (Fig 2A). Modest induction of ELC and SLC mRNAs was also observed. Both basal expression and induction of BLC, SDF-1, ELC and SLC mRNAs were defective in *Ikkα*$^{AA/AA}$ stromal cells, but induction of TNFα and VCAM-1 remained intact or became more efficient. By contrast, very little differences in expression of TNFα-inducible genes were found between WT and *Ikkα*$^{AA/AA}$ stromal cells (Fig 2A).

TNFα induced both rapid and delayed nuclear translocation of RelA in WT and *Ikkα*$^{AA/AA}$ stroma cells (Fig 2B). Induction of RelA nuclear translocation by anti-LTβR was slower than the response to TNFα and was also not affected by the *Ikkα*^{AA} mutation. Neither TNFα nor anti-LTβR had a significant effect on the subcellular distribution of p50, as this NF-κB subunit was constitutively nuclear. Both TNFα and anti-LTβR induced nuclear translocation of RelB in WT cells, but TNFα was capable of sending RelB to the nucleus of *Ikkα*$^{AA/AA}$ cells. As expected, anti-LTβR, but not TNFα, stimulated nuclear entry of p52 and this effect was seen in WT cells (Fig 2B). In WT BMDCs, LTβR engagement led to induction of SLC, ELC and IκBα mRNAs (Fig 2C). SLC and ELC, however, were not induced in BMDC from *Ikkα*$^{AA/AA}$ mice. Again, we found that at least one gene, this time CXCR5, was hyperinducible in mutant cells. As in stromal cells, anti-LTβR induced RelA nuclear translocation in both WT and *Ikkα*$^{AA/AA}$ BMDCs, but its ability to induce p52 and RelB nuclear entry was abolished in *Ikkα*$^{AA/AA}$ cells (Fig 2D).

The results shown above and genetic analysis of NF-κB2 (G. Franzoso *et al.*, *J. Exp. Med.* 187, 147-159 (1998)) and RelB-(D. S. Weih, Z. B. Yilmaz, F. Weih, *J Immunol* 167,

1909-1919 (2001)) deficient mice suggest that *Blc*, *Sdf-1*, *Elc* and *Slc* genes induction requires RelB:p52 nuclear translocation.

EXAMPLE 4

IKKα is required for recruitment of RelB to the *Blc*, *Sdf-1*, *Elc* and *Slc* promoters

To address whether the *Blc*, *Sdf-1*, *Elc* and *Slc* genes are in fact direct targets for RelB-containing dimers and determine whether they are also recognized by RelA-containing dimers, we performed chromatin immunoprecipitation (ChIP) experiments (S. Saccani, G. Natoli, *Genes Dev* 16, 2219-2224 (2002)). In splenic stromal cells, anti-LTβR induced efficient recruitment of RelB, but not RelA, to the *Blc* and *Sdf-1* promoters (Fig 3A). This response was abolished in $Ikk\alpha^{AA/AA}$ cells. Treatment with TNFα also induced RelB recruitment to these promoters, but this response was slower and weaker than the response to anti-LTβR and not affected by the $Ikk\alpha^{AA}$ mutation (Fig 3A). As a control we analyzed the same immunoprecipitates for presence of the *Tnfα* and *Vcam1* promoter regions. We found efficient precipitation of both promoter fragments by anti-RelA antibodies and weak or no signal with anti-RelB (Fig 3A). Recruitment of either Rel protein to these promoters was not IKKα-dependent. We also examined recruitment of the large subunit of RNA polymerase II (Pol II). Importantly, recruitment of Pol II to the *Blc* and *Sdf-1* promoters correlated with recruitment of RelB and was seen in anti-LTβR stimulated WT cells, while recruitment of Pol II to the *Vcam1* and *Tnfα* promoters was IKKα-independent (Fig 3A). In BMDC, treatment with anti-LTβR induced efficient recruitment of RelB, but not RelA, to the *Elc* and *Slc* promoters (Fig 3B). No recruitment of RelA was observed. By contrast, both RelB and RelA were recruited to the *IκBα* promoter (whose activation was IKKα-dependent) in response to either TNFα or anti-LTβR, but neither response was IKKα-dependent (Fig 3B). As observed for RelB, the LTβR-induced recruitment of Pol II to the *Slc* and *Elc* promoters was IKKα-dependent (Fig 3B).

EXAMPLE 5

The *Blc* and *Elc* promoters contain a unique κB site that is selectively recognized by RelB:p52 dimers

Selective recruitment of RelB-containing NF-κB dimers to the *Blc*, *Sdf-1*, *Elc* and *Slc* promoters could reflect, previously unknown, intrinsic differences in sequence selectivity between RelB- and RelA-containing dimers. To examine this possibility, we analyzed binding of NF-κB proteins to the *Blc* promoter. The results are shown in Fig 4.

The proteins referred to in Fig 4 are as follows: RelB RHD (SEQ ID NO:62, *i.e.*, residues 1-400 of GenBank accession A42023 shown in Figure 13), p52 RHD (SEQ ID NO:64, *i.e.*, residues 1-340 of GenBank Accession NM 002502 shown in Figure 8), RelB dimerization domain (DD) (SEQ ID NO:63, *i.e.*, residues 278-378 of GenBank accession A42023 shwon in Figure 13), p50 RHD (residues 1-363), p50 RHD (residues 39-363), (residues 1-363), and RelA RHD (SEQ ID NO:65, *i.e.*, residues 19-291 of GenBank accession M61909 shown in Figure 20).

Several ^{32}P-labeled probes were derived from the 700 base pair (bp) region (-688 to +12) contained within the ChIP primer set (Fig 4A). One of them, covering the region from –191 to –20, exhibited strong binding to recombinant RelB:p52 and weak binding to RelA:p50 dimers. Several other probes (from –770 to –460, -460 to –380 and –380 to –200, as well as –770 to -980) did not bind either dimer. To narrow down the sequence responsible for RelB:p52 binding we generated a shorter probe (Probe 1) covering the region from –191 to –64. This probe exhibited very strong binding to RelB:p52 and weak binding to RelA:p50 (Fig 4B). On the other hand, RelA:p50 and RelB:p52 exhibited little differences in their ability to bind a consensus κB probe, whereas a 200 bp probe (Probe 2) derived from the far 5' upstream region (-1900 to –1700) of the *blc-1* gene was preferentially recognized by RelA:p50 (Fig 4B). Probe 1 (-191 to –64) contains one potential NF-κB binding site. We synthesized two overlapping smaller probes containing this site (Fig 4C) and used them to examine binding of RelA:p50, RelB:p52, as well as RelB:p50. Both probes, which contained the sequence 5'-GGGAGATTTG-3' (SEQ ID NO:59), were efficiently recognized by RelB:p52 and weakly by RelA:p50 (Fig 4B). Binding of RelB:p50 to this probe was barely detectable. To identify whether another IKKα-dependent chemokine gene contains a similar sequence, we have used the Trafac server (A. G. Jegga *et al.*, *Genome Res* 12, 1408-1417 (2002)), which identifies ortholog conserved transcription factor binding sites, to examine human and rodent *Elc* genes. The

putative binding sites were first identified using the MatInspector (Professional Version 4.3,2000) program that utilizes a database of eukaryotic transcription factor binding sites (A. G. Jegga *et al.*, *Genome Res* 12, 1408-1417 (2002)). This procedure identified a very similar sequence to the *Blc*-κB site at positions –64 to –50 of the *Elc* genes (Fig 4C). This site, termed the *Elc*-κB site, was also preferentially recognized by RelB:p52 dimers (Fig 4B).

EXAMPLE 6

Selective, IKKα-dependent, activation of the *Blc* and *Elc* promoters by LTβR engagement

Stimulation of WT MEFs with either TNFα or α-LTβR-induced DNA binding activities recognized by the consensus κB site (Fig 5A). Using the *Blc*-κB and *Elc*-κB sites as probes, we detected induced DNA binding activity in WT MEFs stimulated with anti-LTβR (Fig 5A). This activity was not induced in *Ikkα*$^{-/-}$ MEFs. Similar results were obtained in BMDCs analyzed with the *Elc*-κB probe (Fig 5B).

To examine the function of the newly discovered κB sites, we cloned three copies of either the consensus κB site, the *Blc*-κB site or an inactive version of the latter (m*Blc*-κB) upstream to a minimal SV40 promoter driving a luciferase reporter (Promega). Whereas the consensus κB site conferred inducibility by either TNFα or anti-LTβR, the *Blc*-κB site conferred an efficient response to anti-LTβR but a weak response to TNFα (Fig 5C). The mutated *Blc*-κB site was inactive. While the consensus κB site was equally active in WT and *Ikkα*$^{-/-}$ MEFs, the *Blc*-κB site no longer conferred anti-LTβR responsiveness in *Ikkα*$^{-/-}$ MEFs (Fig 5C). Using the intact *Blc* promoter fused to a luciferase reporter we found efficient induction by anti-LTβR in WT but not in *Ikkα*$^{-/-}$ MEFs. This response was dependent on integrity of the *Blc*-κB site and even its conversion to a consensus κB site attenuated the response to anti-LTβR (Fig 5C). The *Elc* promoter also exhibited preferential activation by anti-LTβR that was IKKα-dependent.

EXAMPLE 7. Materials And Methods

The following is a brief description of additional exemplary materials and methods that may be used in the invention's methods.

A. Primary cell cultures

Stromal cell cultures were established from spleens of WT and *Ikkα*$^{AA/AA}$ mice as described (Skibinski *et al.* (1998) Eur J Immunol, 28, 3940-3948). Spleens were gently ground and released cells cultured in DMEM supplemented with heat-inactivated FCS (Invitrogen, Carlsbad, Ca). After one week, non-adherent cells were removed, adherent cells were washed twice with PBS and cultured one more week in DMEM/FCS. Absence of contaminating myeloid and lymphoid cells was verified by flow cytometry (FACSCalibur, Becton Dickinson). Stromal cells are uniformly positive for ICAM-1. BMDCs were cultured as described (Wu *et al.* (2002) J Immunol, 168, 5096-5102).

B. Adoptive transfers

Bone marrow cells (3-4 x 10^6 cells per mouse) were isolated from femurs of WT or *Ikkα*$^{AA/AA}$ mice and injected intravenously into lethally irradiated recipients. Mice were H-2 matched and, in the case of *Ikkα*$^{AA/AA}$, were from the F3-F5 backcross to C57Bl/6. Mice were provided antibiotics in drinking water and sacrificed 6-8 weeks post reconstitution. When indicated, mice were immunized i.p. with SRBC (Colorado Serum Company, Denver, Co) 7 days prior to sacrifice (Poljak *et al.* (1999) J Immunol, 163, 6581-6588).

C. Immunohistochemical analysis

Cryosections (8 – 10 μM) of spleen were prepared, dried and fixed with acetone before immunohistochemical analysis (Poljak *et al.* (1999) J Immunol, 163, 6581-6588; Weih *et al.* (2001) J Immunol, 167, 1909-1919). Staining reagents were: FDC-M2 (ImmunoKontact, UK), ICAM-1 (Santa-Cruz Biologicals, Ca), B220, and CD35-bio (clone 8C12) (all from BD Pharmingen). Immunecomplexes were detected using species-specific secondary reagents. Sections were viewed by immunofluorescence microscopy (HM505E

Microm Inc, Walldorf, Germany) and images captured with a digital camera (Nikon E800 Scope with Spot Diagnostics Digital Camera, A.G. Heinze Inc., Lake Forest, Ca).

D. Electrophoretic Mobility Shift Assay and Immunoblots

Nuclear and cytoplasmic extracts were prepared and analyzed for levels of NF-κB subunits and DNA binding activity (Bonizzi *et al.* (1999) *et al.* Mol Cell Biol, 19, 1950-1960; Senftleben *et al.* (2001) Science, 293, 1495-1499). Recombinant NF-κB subunits (not full length proteins) were produced in *E.coli* and purified as described (Chen *et al.* (1999) Protein Eng, 12, 423-428). All antibodies and immunoblotting procedures were described (Senftleben *et al.* (2001) Science, 293, 1495-1499).

E. Real Time PCR analysis and Chromatin Immunoprecipitation Assay (ChIP)

Real Time-PCR was performed using a PE Biosystems 5700 thermocycler following the SyBr Green™ protocol. Briefly, 12 ng of total cDNA, 50 nM of each primer and 1x SyBr Green™ mix were used in a total volume of 25 µl. All values were standardized to that of cyclophilin mRNA. Primer sequences are available upon request. ChIP assays were as described (Saccani *et al.* (2002) Genes Dev, 16, 2219-2224). Polyclonal antibodies to p65 (C-20), RelB (C-19) and Pol II (N-19) were from Santa Cruz. The sequences of the promoter-specific primers (*Blc* +12 to –688, *Sdf-1* +22 to -678, *Vcam-1* +30 to -640, *Iκbα* +20 to – 340, *Tnfα* +20 to -545) and a detailed experimental protocol are available upon request.

EXAMPLE 8

Impaired FDC maturation and chemokine production in Stromal cell-derived requires IKKα.

Reconstitution of lethally irradiated mice with *Ikkα*$^{-/-}$ fetal liver hematopoietic progenitors revealed a role for IKKα in late B-cell maturation, splenic organization and germinal center (GC) formation (Kaisho *et al.* (2001) J. Exp. Med., 193, 417-426; Senftleben *et al.* (2001) Science, 293, 1495-1499). However, embryonic lethality precludes the use of *Ikkα*$^{-/-}$ mice to identify functions for IKKα in other cell types involved in spleen development and organization. Homozygous knock-in mice expressing an IKKα variant that cannot be activated (*Ikkα*$^{AA/AA}$ mice) are viable, yet show defects in lymphoid organogenesis and GC formation (Senftleben *et al.* (2001) Science, 293, 1495-1499). Using an antibody against FDC-M2, an FDC (follicular dentritic cell) marker, we found that *Ikkα*$^{AA/AA}$ mice lack mature FDCs (Fig 26A). To identify the cells in which IKKα acts, reciprocal bone marrow chimeras were generated using *Ikkα*$^{AA/AA}$ and WT mice. Six weeks after adoptive transfer, mice were challenged with a T-cell dependent antigen, sheep red blood cells (SRBC), and sacrificed 7 days later. FDC maturation remained impaired in *Ikkα*$^{AA/AA}$ recipients reconstituted with WT bone marrow, whereas a mature FDC network formed in WT recipients reconstituted with *Ikkα*$^{AA/AA}$ bone marrow. Using an antibody against CD35, another FDC marker, we examined formation of mature FDCs, a cell type derived from mesenchymal stromal cells which are important for GC formation. FDC maturation was impaired in *Ikkα*$^{AA/AA}$ recipients reconstituted with WT bone marrow, whereas a mature FDC network formed in WT recipients reconstituted with *Ikkα*$^{AA/AA}$ bone marrow (Fig 26B). These results suggest that IKKα acts in stromal cells of the spleen to induce maturation of FDCs, which are thought to be derived from mesenchymal stromal cells (Fu *et al.* (1999) Annu Rev Immunol, 17, 399-433).

Another aspect of spleen development is segregation of B- and T-lymphocytes to the follicles and the peri-arterial lymphatic sheath (PALS), respectively. WT chimeras reconstituted with *Ikkα*$^{AA/AA}$ bone marrow, but not *Ikkα*$^{AA/AA}$ mice reconstituted with WT bone marrow, exhibited normal B- and T- cell segregation detected by staining with anti-B220 and anti-CD5 antibodies, respectively (Fig 26C). These results also point to a critical action of IKKα in stromal cells, which in addition to giving rise to FDCs, control splenic microarchitecture through production of organogenic chemokines that dictate cell migration and localization (Ansel *et al.* (2001) Curr Opin Immunol, 13, 172-179).

Critical organogenic chemokines for spleen development include: ELC and SLC, ligands for the chemokine receptor CCR7; BLC, which binds CXCR5 (Ansel *et al.* (2000) Nature, 406, 309-314; Forster *et al.* (1999) Cell, 99, 23-33) and SDF-1, which promotes trafficking of both immature and naïve lymphocytes to lymphoid tissues (Kim *et al.* (1999) J Leukoc Biol, 65, 6-15). Previous work revealed that induction of these chemokines in response to engagement of LTβR is defective in *Ikkα*$^{AA/AA}$ mice (Dejardin *et al.* (2002) Immunity, 17, 525-535). We extended these observations to SRBC immunized mice (Fig 26D). Based on previous experiments, we examined the expression of the different genes 48 hrs post-immunization. While induction of the mRNAs for BLC, ELC, SLC and SDF-1 was readily detected in WT spleens, these genes were barely induced in the mutant.

EXAMPLE 9

IKKα is required for LTβR–induced RelB:p52 nuclear translocation and chemokine expression in splenic stromal cells and myeloid dendritic cells.

The defects shown above are very similar to those exhibited by mice lacking LTβR (Fu *et al.* (1999) Annu Rev Immunol, 17, 399-433). The majority of LT®R LTβR expression is restricted to stromal cells of the spleen, however BMDCs have also been

shown to express LT®R LTβR (Browning *et al.* (2002) *et al.* J Immunol, 168, 5079-5087). We therefore isolated and cultured these cellsboth of these cell types from WT and *Ikkα*$^{AA/AA}$ mice. Stimulation of WT stromal cells with agonistic anti-LTβR antibody (Dejardin *et al.* (2002) Immunity, 17, 525-535) resulted in 4-6-fold induction of BLC, SDF-1, TNFα, VCAM-1 and IκBα mRNA (Fig 27A). Modest induction of ELC and SLC mRNAs was also observed. Both basal expression and induction of BLC, SDF-1, ELC and SLC mRNAs were defective in *Ikkα*$^{AA/AA}$ stromal cells. Similar defects in expression of these chemokines in *RelB*$^{-/-}$ and *Nfkb2*$^{-/-}$ mice have been described in *RelB*$^{-/-}$ and *Nfkb2*$^{-/-}$ mice (Poljak *et al.* (1999) J Immunol, 163, 6581-6588; Weih *et al.* (2001) J Immunol, 167, 1909-1919). Moreover, induction of TNFα, IκBα and VCAM-1 in *Ikkα*$^{AA/AA}$ stromal cells remained intact or was even elevated. The increased expression of VCAM-1 could be related to the defective nuclear entry of RelB in *Ikkα*$^{AA/AA}$ cells (see below), as RelB-deficiency was previously found to increase the expression of certain inflammatory genes (Xia *et al.* (1999) Mol Cell Biol, 19, 7688-7696). By contrast, very few differences in expression of TNFα-inducible genes were found between WT and *Ikkα*$^{AA/AA}$ stromal cells (Fig 27A). Unlike anti-LTβR, TNFα was a poor activator of the organogenic chemokines, but was a potent activator of TNFα, IκBα and VCAM-1.

TNFα induced both rapid and delayed nuclear translocation of RelA in WT and *Ikkα*$^{AA/AA}$ stromal cells (Fig 27B). This response was not considerably different in *Ikkα*$^{AA/AA}$ cells (Fig 27B, right panel). Neither TNFα nor anti-LTβR had a significant effect on the subcellular distribution of p50, as this NF-κB subunit was constitutively nuclear (Fig 27B). Both TNFα and anti-LTβR induced nuclear translocation of RelB in WT cells, but TNFα was capable of sending RelB to the nucleus of *Ikkα*$^{AA/AA}$ cells (Fig 27B). In either case, the nuclear translocation of RelB is delayed relative to that of RelA. As expected, anti-LTβR,

but not TNFα, stimulated nuclear entry of p52 and this effect was seen in WT cells (Fig 27B). Similar results in regards to both gene expression and nuclear translocation of NF-κB subunits was observed in BMDCs. In WT BMDCs, LTβR engagement led to induction of SLC, ELC and IκBα mRNA (Fig 27C).

However, SLC and ELC were not induced in BMDC from *Ikkα*$^{AA/AA}$ mice. Again, we found that at least one gene, in this case CXCR5, was elevated in mutant cells. Whereas engagement of LTβR resulted in nuclear entry of RelB and p52 in WT BMDCs, this response was defective in *Ikkα*$^{AA/AA}$ cells (Fig 27D). Nuclear translocation of RelA was not affected in *Ikkα*$^{AA/AA}$ cells. These results and the previous genetic analysis of NF-κB2- (Poljak *et al.* (1999) J Immunol, 163, 6581-6588) and RelB- (Weih *et al.* (2001) J Immunol, 167, 1909-1919) deficient mice strongly suggest that *Blc*, *Sdf-1*, *Elc* and *Slc* gene induction requires RelB:p52 nuclear translocation. Curiously, the induction of RelB and p52 nuclear entry following LTβR engagement was considerably faster in BMDCs than in splenic stromal cells. This is likely to be related to the different origins of these cell types and/or the expression of different levels of LTβR molecules on their surface.

EXAMPLE 10

IKKα is required for recruitment of RelB to the *Blc*, *Sdf-1*, *Elc* and *Slc* promoters.

To address whether the IKKα-dependent genes are in fact direct targets for RelB-containing dimers and whether they are also recognized by RelA-containing dimers, we performed chromatin immunoprecipitation (ChIP) experiments (Saccani *et al.* (2002) Genes Dev, 16, 2219-2224). In splenic stromal cells, anti-LTβR induced efficient recruitment of RelB, but not RelA, to the *Blc* and *Sdf-1* promoters (Fig 28A), which encode the two organogenic chemokines that are most efficiently expressed by these cells (Cyster *et al.*

(2003) Immunol Rev, 195, 5-14). As previously shown, recruitment of NF-κB subunits to promoter DNA may be detected at earlier time points than the ones revealed by immunoblot analysis of nuclear translocation, due to the increased sensitivity of the ChIP assay (Saccani *et al.* (2001) J Exp Med, 193, 1351-1359). Anti-LTβR-induced recruitment of RelB to target gene promoters was abolished in $Ikk\alpha^{AA/AA}$ cells.

However, TNFα-induced RelB promoter recruitment, which was slower and weaker than the response elicited by anti-LTβR, was not affected by the $Ikk\alpha^{AA}$ mutation (Fig 28A). The response to TNFα may depend on formation of RelB:p50 dimers. As a control we analyzed the same immunoprecipitates for the presence of the *Tnfα* and *Vcam1* promoter regions. Efficient precipitation of both promoter fragments by anti-RelA antibodies was shown with a weak signal obtained with anti-RelB (Fig 28A). Recruitment of either Rel protein to these promoters was not IKKα-dependent. Importantly, recruitment of Pol II to the *Blc* and *Sdf-1* promoters correlated with recruitment of RelB and was seen in anti-LTβR stimulated WT cells (Fig 28A).

As mentioned above, splenic stromal cells are the major source of production of BLC and SDF, while BMDCs are a major source of ELC and SLC (Cyster *et al.* (2003) Immunol Rev, 195, 5-14). Therefore, in BMDCs we examined recruitment of the different NF-κB subunits in response to LTβ signaling to the *Elc* and *Slc* promoters. Treatment with anti-LTβR induced efficient recruitment of RelB, but not RelA, to the *Elc* and *Slc* promoters (Fig 28B). No [Little] recruitment of RelA was observed. By contrast, both RelB and RelA were recruited to the *IκBα* promoter in response to either TNFα or anti-LTβR, but neither response was IKKα-dependent (Fig 28B). As observed for RelB, the LTβR-induced recruitment of Pol II to the *Slc* and *Elc* promoters was IKKα-dependent (Fig 28B).

EXAMPLE 11

The *Blc* and *Elc* promoters contain a unique κB site that is selectively recognized by RelB:p52 dimers.

Selective recruitment of RelB-containing NF-κB dimers to the *Blc, Sdf-1, Elc* and *Slc* promoters could reflect, previously unknown, intrinsic differences in sequence selectivity between RelB- and RelA-containing dimers. To examine this possibility, we analyzed binding of NF-κB proteins to the *Blc and Elc* promoters. In this experiment we used truncated recombinant NF-κB proteins to generate NF-κB dimers of known composition. All of the proteins used in these experiments were fully characterized and even crystalized (Ghosh *et al.* (1995) Nature, 373, 303-310; Chen *et al.* (1999) Protein Eng, 12, 423-428; G. Ghosh, unpublished data). Several ^{32}P-labeled probes were derived from the 700 base pair (bp) proximal region (-688 to +12) of the *Blc* promoter, contained within the ChIP primer set (Fig 29A). One of the probes, spanning positions –191 to –20, exhibited strong binding to recombinant RelB:p52 and weak binding to RelA:p50 dimers. Several other probes (from –770 to –460, -460 to –380 and –380 to –150, as well as from –770 to -980) did not detectably bind either dimer. To narrow down the sequence responsible for RelB:p52 binding we generated a shorter probe (Probe 1) covering the region from –191 to –64. This probe exhibited very strong binding to recombinant RelB:p52 and weak binding to RelA:p50 (Fig 29B). On the other hand, the RelA:p50 and RelB:p52 dimers exhibited little differences in their ability to bind a consensus κB probe, whereas a 200 bp probe (Probe 2) derived from the far 5' upstream region (-1900 to –1700) of the *Blc* gene was preferentially recognized by RelA:p50. Probe 1 (-191 to –64) contained one potential NF-κB binding site. We synthesized two overlapping smaller probes containing this site (Fig 29C) and used them to examine binding of RelA:p50, RelB:p52, as well as RelB:p50. Both probes, which contained the sequence 5'-GGGAGATTTG-3' (SEQ

ID NO:59), were efficiently recognized by RelB:p52 and weakly by RelA:p50 (Fig 29B). Binding of RelB:p50 to these probes was barely detectable. In all cases, the detected protein-DNA complexes were specific as indicated by competition experiments.

To identify whether another IKKα-dependent chemokine genes contain a similar sequence, we used the Trafac server (Jegga *et al.* (2002) Genome Res, 12, 1408-1417), which identifies ortholog conserved transcription factor binding sites, to examine the human and rodent *Elc* genes. The putative binding sites were first identified using the MatInspector program (Professional Version 4.3,2000) that utilizes a database of eukaryotic transcription factor binding sites (Jegga *et al.* (2002) Genome Res, 12, 1408-1417). This procedure identified a very similar sequence to the *Blc*-κB site at positions –64 to –50 of the *Elc* genes (Fig 29C). This site, termed the *Elc*-κB site, was also preferentially recognized by RelB:p52 dimers (Fig 29B).

EXAMPLE 12

Selective, IKKα-dependent, activation of the *Blc* and *Elc* promoters by LTβR engagement and IKKα-dependent induction of *Rxra*, *Irf3* and *Baff* mRNAs.

We next used MEFs, which unlike the related stromal cells are amenable to transfection (Bebien M., unpublished results), to examine the function of the RelB:p52 specific sites. Stimulation of WT MEFs with either TNFα or α-LTβR-induced DNA binding activities recognized by the consensus κB site (Fig 30A). Using the *Blc*-κB and *Elc*-κB sites as probes, we detected induced DNA binding activity in WT MEFs stimulated with anti-LTβR (Fig 30A). This activity was not induced in *Ikkα*$^{-/-}$ MEFs. Similar results were obtained in BMDCs analyzed with the *Elc*-κB probe (Fig 30B). Next, we cloned three copies of either the consensus κB site, the *Blc*-κB site or an inactive version of the latter (m*Blc*-κB) upstream to a minimal SV40 promoter driving a luciferase reporter and

transfected the constructs into WT and *Ikkα*$^{-/-}$ MEFs. The consensus κB site conferred inducibility by either TNFα or anti-LTβR, whereas the *Blc*-κB site conferred an efficient response to anti-LTβR but a weak response to TNFα and the mutated *Blc*-κB site was inactive (Fig 30C). While the consensus κB site was equally active in WT and *Ikkα*$^{-/-}$ MEFs, the *Blc*-κB site did not confer anti-LTβR responsiveness in *Ikkα*$^{-/-}$ MEFs (Fig 30C). Using the intact *Blc* promoter fused to a luciferase reporter we found efficient induction by anti-LTβR in WT but not in *Ikkα*$^{-/-}$ MEFs. This response was dependent on the integrity of the *Blc*-κB site and even its conversion to a consensus κB site attenuated the response to anti-LTβR (Fig 30C). The *Elc* promoter also exhibited preferential activation by anti-LTβR that was IKKα-dependent.

To further examine the relevance of the RelB:p52 selective binding site, we conducted a pattern search with two strings, namely AGGAGATTTG (*Elc*-κB) (SEQ ID NO:60) and GGGAGATTTG (*Blc*-κB) (SEQ ID NO:59) using the Trafac server and the BlastZ algorithm (Http://bio.cse.psu.edu). Closely similar (at least 8/10 identity) sites were detected within 5 kb upstream to the start sites of the *Sdf-1* and *Baff* genes, whose expression is known to be *Ikkα*-dependent (Dejardin *et al.* (2002) Immunity, 17, 525-535) (Figs 26D, 27A, 30D). We also detected similar and evolutionary conserved sites with the same region of several other genes, whose IKKα-dependence was previously unknown (Fig 30D). RT-PCR analysis revealed that two of these genes, *Rxra* and *Irf3*, coding for important transcription factors, were induced in stromal cells in response to anti-LTβR in a manner dependent on IKKα (Fig 30E).

From the above, it is clear that the invetion provides nucleotide sequences that mediate one or more functions of IKKα, kits and methods for using these sequences to identify therapeutic compounds that alter IKKα related pathology.

All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiment, it should be understood that the invention as claimed should not be unduly limited to such specific embodiment. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in the art and in fields related thereto are intended to be within the scope of the claims.